DEVELOPMENT AND VALIDATION OF A UPLG ASSAY METHOD FOR THE SIMULATIONS ESTIMATION OF PAROXETINE AND CLONAZEPAM IN TABLET DOSAGE FORM.

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INTRODUCTION

ANALYTICAL CHEMISTRY

Analytical chemistry is defined as the science and art of developing sensitive, reliable and accurate method for determining the composition of materials in terms of elements or compounds which they contain. Pharmaceutical analysis comprises those procedures necessary to determine the identity, strength, quality and purity of drug and chemicals Pharmaceutical analysis deals mainly with bulk materials, dosage forms and more recently, biological samples in support of bio-pharmaceutical and pharmacokinetic studies.

Pharmaceutical analysts in research and development (R&D) of pharmaceutical industry plays a very comprehensive role in new drug development and follow up activities to assure that a new drug product meets the established standards, its stability and continued to meet the purported quality throughout its shelf life. The different activity of R&D includes drug development, (synthesis and manufacture), formulation, clinical trials, evaluations and finally launching i.e. finished products. Closely associated with these are regulatory and quality assurance functions.**(Jary D.Chiristan 5th Edition)**

CHROMATOGRAPHY AND ITS TYPES

A variety of analytical techniques such as spectroscopy (UV-Visible), gas chromatography (GC), high performance liquid chromatography (HPLC),Ultra performance liquid chromatography (UPLC) supercritical fluid chromatography (SFC), capillary electrophoresis (CE) coupled with selective detectors (diode-array detector (DAD) and mass spectrometry (MS)) are frequently used to achieve the above requirements

In spite of various techniques available, HPLC and UPLC has become a universal tool for pharmaceutical and biomedical research, as well as product analysis. The availability of fully automated systems, excellent quantitative precision, accuracy, sensitivity, selectivity, increased selection of column stationary phases, applicability to a broad variety of sample matrices and ability to hyphenate with several spectroscopic detectors has made HPLC or UPLC the instrument of choice for the analysis of most categories of drugs.

Similarly, HPLC or UPLC methods are abundantly used in the field of biomedical analysis, viz. therapeutic drug monitoring, pharmacokinetic and bioequivalence studies. The assay of drugs in blood, plasma and tissues presents analytical challenges. The drug substance is typically present at low concentrations, bound to proteinaceous material and endogenous compounds typically present in the samples can interfere with the analysis. For these reasons, the analytical methods usually be highly sensitive to detect analytes at low concentrations and required a sample pretreatment procedure such as liquid-liquid extraction (LLE) or solid phase extraction (SPE), to isolate the analyte from the complex biological matrix. Hence, high sensitivity and automation of sample processing tools to deal with large number of samples are strong incentives for the consideration of HPLC or UPLC methods in biomedical analysis.

The number of drugs introduced into the market is increasing every year, these drugs may be either new entities or partial structural modifications of the existing ones. Very often, there is a time lag from the date of introduction of a drug into the market to the date of its inclusion in the pharmacopoeias. This happens because of the possible uncertainties in the continuous and wider usage of these drugs, reports of new toxicities (resulting in their withdrawal from the market), development of patient resistance and introduction of better drugs by competitors. Under these conditions, standards and analytical procedures for thesedrugs may not be

available in pharmacopoeias. It becomes necessary, therefore, to develop newer analytical methods for such drugs.

UPLC comes from HPLC .Ultra Performance Liquid Chromatography (UPLC) is a relatively new technique giving new possibilities in liquidchromatography, especially concerning decrease of time and solvent consumption.This enhanced the demand for UPLC methods for the simultaneous determination of drugs in pharmaceutical mixtures.

INTRODUCTION TO HIGH PERFORMANCE LC

The acronym HPLC, coined by the late Prof.Csaba Horvath for his 1970 Pittcon paper,originally indicated the fact that high pressure was used to generate the flow required forliquid chromatography in packed columns. In the beginning, pumps only had a pressurecapability of 500 psi. This was called high pressure liquid chromatography, or HPLC. **(Gurdeep RChathwal, Sham K Anand, 2.624-2-631)**

The early 1970s saw a tremendous leap in technology. These new HPLC instruments could develop up to 6,000 psi of pressure, and incorporated improved injectors, detectors, and columns. HPLC really began to take hold in the mid-to late-1970s. With continued advances in performance during this time (smaller particles, even higher pressure), the acronym HPLC remained the same, but the name was changed to high performance liquid chromatography.

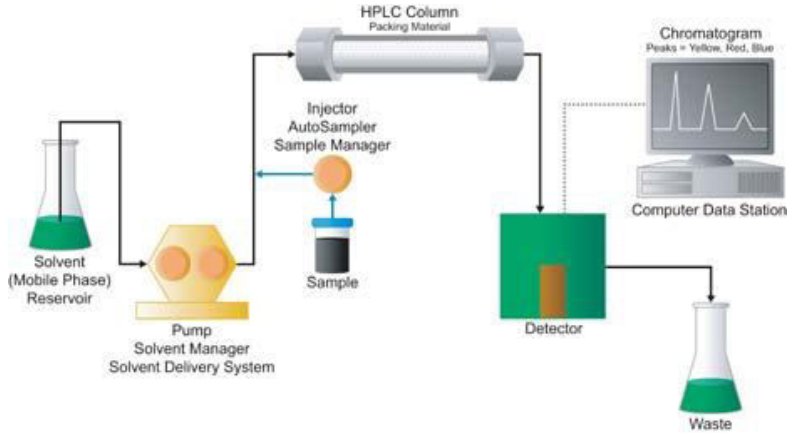


Figure 1 High-Performance Liquid Chromatography [HPLC] System

High performance liquid chromatography is now one of the most powerful tools in analytical chemistry. It has the ability to separate, identify, and quantitate the compounds that are present in any sample that can be dissolved in a liquid. Today, compounds in trace concentrations as low as parts per trillion (ppt) may easily be identified. HPLC can be, and has been, applied to just about any sample, such as pharmaceuticals, food, nutraceuticals, cosmetics, environmental matrices, forensic samples, and industrial chemicals. The components of a basic high-performance liquid chromatography (HPLC)system are shown in the simple diagram in Figure 1 .

A reservoir (Solvent Delivery) holds the solvent (called the mobile phase, because it moves). A high-pressure pump solvent manager is used to generate and meter a specified flow rate of mobile phase, typically millilitres per minute.

An injector (sample manager or auto sampler) is able to introduce (inject) the sample into the continuously flowing mobile phase stream that carries the sample into the HPLC column. The column contains the chromatographic packing material needed to effect the separation. This packing material is called the stationary phase because it is held in place by the column hardware. A detector is needed to see the separated compound bands as they elute from the HPLC column (most compounds have no color, so we cannot see them with our eyes).

The mobile phase exits the detector and can be sent to waste, or collected, as desired. When the mobile phase contains a separated compound band, HPLC provides the ability to collect this fraction of the eluate containing that purified compound for further study. This is called preparative chromatography. The high-pressure tubing and fittings are used to interconnect the pump, injector, column, and detector components to form the conduit for the mobile phase, sample, and separated compound bands.

The detector is wired to the computer data station, the HPLC system component that records the electrical signal needed to generate the chromatogram on its display and to identify and quantitate the concentration of the sample constituents. Since sample compound characteristics can be very different, several types of detectors have been developed. For example, if a compound can absorb ultraviolet light, a UV-absorbance detector is used. If the compound fluoresces, a fluorescence detector is used. If the compound does not have either of these characteristics, a more universal type of detector is used, such as an evaporative-light-scattering detector (ELSD). The most powerful approach is the use multiple detectors in series. For example, a UV and/or ELSD detector may be used in combination with a mass spectrometer (MS) to analyse the results of the chromatographic separation. This provides, from a single injection, more comprehensive information about an analyse. The practice of coupling a mass spectrometer to an HPLC system is called LC/MS.

INTRODUCTION TO ULTRA PERFORMANCE LIQUID CHROMATOGRAPHY

In 2004, further advances in instrumentation and column technology were made to achieve very significant increases in resolution, speed, and sensitivity in liquid chromatography. Columns with smaller particles (1.7 micron) and instrumentation with specialized capabilities designed to deliver mobile phase at 15,000 psi (1,000 bars) were needed to achieve a new level of performance. A new system had to be holistically created to perform ultra-performance liquid chromatography, now known as UPLC technology.

Basic research is being conducted today by scientists working with columns containing even smaller 1-micron-diameter particles and instrumentation capable of performing at100,000 psi. This provides a glimpse of what we may expect in the future. UPLC refers to Ultra Performance Liquid Chromatography. It improves in three areas: chromatographic resolution, speed and sensitivity analysis. It uses fine particles and saves time and reduces solvent

consumption . UPLC is comes from HPLC. HPLC has been the evolution of the packing materials used to effect the separation. An underlying principle of HPLC dictates that as column packing particle size decreases, efficiency and thus resolution also increases. As particle size decreases to less than 2.5µm, there is a significant gain in efficiency and it's doesn't diminish at increased linear velocities or flow rates according to the common Van Demeter equation . By using smaller

particles, speed and peak capacity (number of peaks resolved per unit time) can be extended to new limits which is known as Ultra Performance.

The classic separation method is of HPLC (High Performance Liquid Chromatography) with many advantages like robustness, ease of use, good selectivity and adjustable sensitivity. Its main limitation is the lack of efficiency compared to gas chromatography or the capillary electrophoresis due to low diffusion coefficients in liquid phase, involving slow diffusion of analyses in the stationary phase. The Van Demeter equation shows that efficiency increases with the use of smaller size particles but this leads to a rapid increase in back pressure, while most of the HPLC system can operate only up to 400 bar. That is why short columns filled with particles of about 2µm are used with these systems, to accelerate the analysis without loss of efficiency, while maintaining an acceptable loss of load.

To improve the efficiency of HPLC separations, the following can be done,

(1) Work at higher temperatures

(2) Use of monolithic columns **Swatrz M.E 2005**

METHOD DEVELOPMENT AND OPTIMIZATION OF CHROMATOGRAPHIC CONDITIONS

Methods for analysing drugs in multi component dosage forms can be developed if the nature of the sample, namely, its molecular weight, polarity, ionic character and the solubility parameter are in hand. Until and unless considerable trial and error procedures have not performed, an exact recipe for HPLC cannot be provided. The most difficult problem usually is where to start, what type of column is worth trying with what kind of mobile phase. In general one begins with reversed phase chromatography, when the compounds are hydrophilic in nature with many polar groups and are water soluble.(**Skoog et al 2005)**.

Selection of stationary phase/ column:

Selection of the column is the initial and the most noteworthy step in method development. The proper choice of separation column includes three different approaches. 1. Selection of separation system 2. The particle size and the nature of the column packing**3**. The physical parameters of the column i.e. the length and the diameter. Some of the crucial parameters considered while selecting chromatographic Columns. They are length and diameter of the column, packing material, Shape of the particle, Size of the particles, % of Carbon loading, Pore volume, Surface area and End capping. The column is selected depending on the nature of the solute and the information about the analyse. Reversed phase mode of chromatography facilitates information about the analyse. Reversed phase mode of chromatography facilitates a wide range of columns like dimethyl silane (C2), butyls lane (C4), octylsilane (C8), octadecylsilane (C18), base deactivated silane (C18) BDS, cyan propyl(CN), nitro, amino etc.

Selection of mobile phase:

The primary objective in selection and optimization of mobile phase is to achieve optimum separation of all the individual impurities and degrades from one other and from analyse peak. In liquid chromatography, the solute retention is governed by the solute distribution factor, which reflects the different interactions of the solute-stationary phase, solute-mobile phase and the mobile phase-stationary phase. For a given stationary phase, the retention of the given solute depends directly upon the mobile phase, the nature and the composition of which has to be judiciously selected in order to get appropriate and required solute retention. The mobile phase has to be adapted in terms of elution strength (solute retention) and solvent selectivity (solute separation). Solvent polarity is the key word in chromatographic separations since a polar mobile phase will give rise to low solute retention in normal phase and high solute retention in reverse phase LC. The selectivity will be particularly altered if the buffer pH is close to the pKa of the analytes; the solvent strength is a measure of its ability to pull analyte from the column. It is generally controlled by the concentration of the solvent with the highest strength. Buffer, pH of the buffer ,mobile phase composition are the parameters, which shall be taken into consideration while selecting and optimizing the mobile phase. Firstly, Buffer and its strength play an important role in deciding the peak symmetries and separations. Some of the most, commonly employed buffers are Phosphate buffer (Potassium dihydrogen phosphate, Dipotasium hydrogen phosphate, Sodium dihydrogen phosphate, Disodium hydrogen phosphate), Phosphoric acid buffers prepared using O-Phosphoric acid, Acetate buffers (Ammonium acetate, Sodium acetate) and Acetic acid buffers prepared using acetic acid. The retention time also depends on the molar strengths of the buffer. Molar strength is increasingly proportional to retention

times. The strength of the buffer can be increased, if necessary, to achieve the required separations. The solvent strength is a measure of its ability to pull analytes from the column. It is generally controlled by the concentration of the solvent with the highest strength.

Secondly, pH plays an important role in achieving the chromatographic separations asit controls the elution properties by controlling the ionization characteristics. Experiments were conducted using buffers having different pH to obtain the required separations. It is important to maintain the pH of the mobile phase in the range of 2.0 to8.0 as most columns does not withstand the pH which are outside this range. Thisis due to the fact that the siloxane linkage area gets cleaved below pH 2.0, whilepH valued above 8.0 silica may dissolve.

Finally, by choosing the optimum mobile phase composition most of the chromatographic separations can be achieved This is due to the fact that fairly large amount of selectivity can be achieved by choosing the qualitative and quantitative composition of aqueous and organic portions. Most widely used solvents in reverse phase chromatography are methanol and acetonitrile. Experiments were conducted with mobile phases having buffers with different pH and different organic phases to check for the best separations between the impurities. A mobile phase which gives separation of all the impurities and degrades from the analyte peak and which is rugged with variations of both aqueous and organic phase by at least ±0.2%.

The low solubility of the sample in the mobile phase can also cause bad peak shapes. It is always advisable to use the same solvents for the preparation of sample solution for the preparation of sample solution as the mobile phase to avoid precipitation of the compounds in the column injector.

Optimizations are often started only after a reasonable chromatogram has been obtained. A reasonable chromatogram means that more or less symmetrical peaks on the chromatogram detect all the compounds. By slight modification of the mobile phase composition, the position of the peaks can be predicted within the range of investigated changes. An optimized chromatogram is the one in which all the peaks are symmetrical and are well separated in less run time.

VALIDATION

The word "validation" means "Assessment" of validity or action of validity or action of providing effectiveness'.

Definitions

FDA defines validation as "establish the documented evidence which provides a high of assurance that a specific process will consistently produce a product of predetermined specifications and quantity attributes".

WHO action of providing that, any procedure, process, equipment, material, activity, or system actually leads to the expected results.

EUMGP define validation as "action of proving in accordance with the principle of Good manufacturing practice (GMP), that any material, activity or system actually lead to expected result".

AUSTRALIANGMP defines validation as "the action of proving that any material, process, activity, procedure, system, equipment or mechanism and intended results".

METHOD VALIDATION GUIDELINES

Regulatory agencies such as the International Conference on Harmonization of Technical Requirements for Registration of Pharmaceuticals for Human Use (ICH), the Food and Drug Administration (FDA) and the United States Pharmacopoeia (USP) in the European community, Japan and United States have published a number of guidelines to assist pharmaceutical companies in validation of analytical methods for drug substances.

FDA guidelines

The FDA published the draft on "Guidance for Industry on Analytical Procedures and Method Validation" to aid pharmaceutical companies in meeting the code of federal regulations requirement [FDA, 2000]. This guidance, when approved, supersedes the FDA Guidance for Industry on Submitting Samples and Analytical Data for Method Validation [FDA, 1987]. According to the FDA, analytical methods are categorized into four tests: identification, testing for impurities (quantitative and limit), assay (dissolution, content, potency), and specific tests. Depending on the type of test, different validation requirements may apply because method validation requirements are directly related to the purpose of the analytical procedure. Recently, the Centre for Drug Evaluation and Research (CDER), a division of the FDA, also issued guidance for validation of bio analytical methods [FDA-CDER, 2001].

ICH guidelines

The ICH was initiated around 1990. It is an important regulatory initiative to standardize regulatory requirements between the European Community, Japan and the United States. Recognizing the benefits of having consistent international requirements, ICH

developed a number of guidelines that have been recognized in these countries. The two main documents of ICH guidelines pertaining to analytical method validation are:

(i) Q2A: Text on Validation of Analytical Procedures [ICH-Q2A, 1995]

(ii) Q2B: Validation of Analytical Procedure Methodology [ICH-Q2B, 1997]

USP guidelines

The USP [USP, 2006] categorizes analytical methods into four types of tests: quantitation of major components of drug product, testing for impurities (quantitative and limit), performance characteristics and identification tests. USP refers to the same definitions of ICH Q2A and Q2B recommendations for procedures on meeting validation requirements. The difference in USP and ICH terminology is for the most part one of semantics; however, there is one notable exception. ICH treats system suitability as a part of method validation, whereas the USP treats it in a separate chapter (621).

Table 1 Comparison of validation parameters required for HPLC assay methods

ICH Guidelines	USP Guidelines	FDA Guidelines
Accuracy	Accuracy	Accuracy
Precision	Precision	Precision
Repeatability	--	Repeatability
Inter-day precision	--	Inter-day precision
Reproducibility	--	Reproducibility
Specificity	Specificity	Specificity
Limit of detection	Limit of detection	Limit of detection
Limit of quantification	Limit of quantification	Limit of quantification

Linearity	Linearity	Linearity
Range	Range	Range
--	Ruggedness	--
Robustness	Robustness	Robustness
System suitability	System suitability	System suitability

Analytical method validation

Method validation is the process for establishing that performance characteristics of the analytical method are suitable for the intended application. Chromatographic methods need to be validation before first routine use. To obtain the most accurate results, all of the variables of the method should be considered, including sampling procedure, sample preparation, chromatographic separation, detection and data evaluation, using the same matrix as that of the intended sample. The validity of an analytical method can only be verified by laboratory studies. All validation experiments used to make claims or conclusions about validity of the method should be documented in report.

Types of analytical procedures to be validated

- Identification test for impurities
- Quantitative test for impurities
- Limit test control of impurities
- Quantitative test for the active moiety in samples of drug substance or drug product, or other selected components (s) in the drug product.
- Dissolution testing.

VALIDATION PARAMETERS AS PER ICH & FDA GUIDELINES

(i) Accuracy

The accuracy of an analytical procedure expresses the closeness of agreement between the value, which is accepted either as a conventional true value or an accepted reference value and the value found.

The accuracy may be determined by application of analytical method to an analyte of known purity (example: reference standard) and also by comparing the results of the method with those obtained using an alternate procedure that has been already validated.

The true value for accuracy assessment can be obtained in several ways and the value found as:

One method is to compare the results of the method with results from an established reference method. This approach assumes that the uncertainty of the reference method is known.

Secondly, accuracy can be assessed by analyzing a sample with known concentrations and comparing the measured value with the true value as supplied with the material.

Third method if certified reference materials or control samples are not available, a blank sample matrix of interest can be spiked with a known concentration by weight or volume. After extraction of the analytic from the matrix and injection into the analytical instrument, its recovery can be determined by comparing the response of the extract with the response of the reference material dissolved in a pure solvent. Because this accuracy assessment measures the effectiveness of sample preparation, care should be taken to mimic the actual sample preparation as closely as possible. At each recommended level studied, replicate samples are evaluated. Accuracy should be assessed using a minimum of 9

determinations over a minimum of 3 concentrations (3 replicates each of the total analytical procedure). Accuracy studies for drug substance and drug product are recommended to be performed at the 80, 100 and 120% levels of label claim as stated in the Guidance for submitting samples and analytical data for methods Validation and as well the accuracy should be within the range 98-102%.

(ii) Precision

The precision of an analytical procedure expresses the closeness of agreement between a series of measurement obtained from multiple sampling of the same homogenous sample under the prescribed conditions. Precision of an analytical procedure is usually expressed the variance, standard deviation of coefficient of variation of a series of measurement.

Precision may be considered at three levels: repeatability, intermediate precision and reproducibility. Precision should be investigated using homogeneous, authentic samples. However, if it is not possible to obtain a homogeneous sample it may be investigated using artificially prepared samples or a sample solution.

For a good method precision, the intra-day and inter-day precisions should be within the acceptance criteria of % RSD $\leq$2.0.respectively.

(iii) Specificity

Specificity is the ability to assess unequivocally the analyte in the presence of components, which may be expected to be present. Typically, these might include impurities, degrades, matrix, etc. For formulation assay, a sample matrix may include impurities, degradation products, excess raw materials, or excipients. The chromatographic method

should be specific and sensitive as required for all known relevant degradation products and/or impurities.

(iv) Limit of detection & Limit of quantification

The LOD of an individual analytical procedure is the lowest amount of analyte in a sample, which can be detected but not necessarily quantitated as an exact value. The LOQ of an individual analytical procedure is the lowest amount of analyte in a sample, which can be quantitatively determined with suitable precision and accuracy. The typical methods recommended by ICH to determine the LOD and LOQ are:

(i) Signal-to-noise ratio: The LOD and LOQ can be expressed as a concentration at a specified signal-to-noise ratio obtained from samples spiked with analyte.

(ii) Standard deviation of the response and the slope of the calibration curve(s) at levels approximating the LOD: LOD was defined as $3.3\sigma/S$ and LOQ as $10\sigma/S$, where S is the slope of the calibration curve and σ is the standard deviation that can be determined based on the standard deviation of the blank, on the residual standard deviation of the regression line, or the standard deviation of y-intercepts of the regression lines.

(v)Linearity

Linearity is the method to obtained tests that are directly proportional to the analyte concentration within a given range.

A range of standards should be prepared containing at least 5 different concentrations of analyte, which are approximately evenly spaced, and span 80-120% of the label claim.

Acceptance criteria:

Correlation coefficient should be not less than 0.9990.

% of y-intercept should be ±2.0. % of RSD for level 1 and level 5 should be not more than±2.0

(vi)Ruggedness

Ruggedness is the degree of reproducibility of test results obtained by the analysis of the same samples under a variety of test conditions such as different laboratories, analysis, instruments, reagent lots, elapsed assay times, temperature, days etc.

It can be expressed as lack influence of the operation and environmental variable on the test results of the analytical method.

(vii)Robustness

It is measure of capacity of an assay to remain unaffected by small but deliberate variation in method parameters and provide an indication of its reliability in normal usage degradation and variation in chromatography columns, mobile phase and inadequate method development are common causes of lack of robustness.

ABBREVIATIONS

%	:	Percentage
Nm	:	Nanometre
V/V	:	Volume by Volume
Min	:	Minute
Mg	:	Milligram
nag	:	Nano gram
TEA	:	Triethyl amine
LC	:	Liquid Chromatography
HPLC	:	High-Performance Liquid Chromatography
GC	:	Gas Chromatography
UV	:	Ultraviolet
HPTLC	:	High –Performance Thin Layer Chromatography
TLC	:	Thin Layer Chromatography
IPC	:	Ion Pair Chromatography
RPIPC	:	Reverse Phase Ion- Pair Chromatography
LC-MS	:	Liquid Chromatography-Mass Spectroscopy
FLU	:	Fluorescence
EC	:	Electrochemical
mm	:	Millimetre
RSD	:	Relative Standard Deviation
RP-HPLC	:	Reverse Phase-High-Performance Liquid Chromatography
RI	:	Relative Index
RRT	:	Relative Retention Time
USP	:	United States Pharmacopoeia

HETP	:	High Equivalent Theoretical Plate
LOD	:	Limit of Detection
LOQ	:	Limit of Quantification
CV	:	Coefficient Variance
NMT	:	Not More Than
NLT	:	Not Less Than
R^2	:	Correlation Coefficient
ACN	:	Acetonitrile
ICH	:	International Conference of Harmonization
Rt	:	Retention time
S/N	:	Signal to Noise
PDA	:	Photo Diode Array Detector
USFDA	:	United State Food and Drug Administration
PPM	:	Parts Per Million
WHO	:	World Health Organization
LQC	:	Low Quality Control
MQC	:	Medium Quality Control
HQC	:	High Quality Control
UPLC	:	Ultra Performance Liquid Chromatography

AIM AND PLAN OF WORK

Aim

The aim of the present research work is to develop a new analytical method for the simultaneous estimation of paroxetine and clonazepam in tablet dosage form by Ultra Performance Liquid Chromatography [UPLC].

Objective

The literature survey reveals that there are several methods reported for the estimation of paroxetine and clonazepam alone or in combination with other drugs in their pharmaceutical dosage forms but none of the method available for the estimation of these drugs in the selected pharmaceutical dosage form.

It is not uncommon to administer two or more drugs in a single formulation, it may be to reduce the number of medicaments to be taken at a time for better patient compliance, to have broad spectrum of activity as in the case of formulations containing antimicrobial agent, for synergic effect or potentiating effect to reduce side effects or for quick relief. It is a challenging task for the analyst to develop a simple analytical method for simultaneous estimation of multiple drug formulations with desired degree of accuracy and precision. In the present work attempts have been made to develop Rapid UPLC method of analysis for a simultaneous estimation of selected two drugs in tablet dosage form.

Hence on the basis of the literature survey, it was thought to develop a precise, accurate, simple, rapid and reliable method for estimation of paroxetine and clonazepam drug in tablets using the following technique of UPLC.

Plan of work was designed as follows:

- Literature Survey
- Study of physicochemical properties of drug (pH, pKa and solubility).
- Procurement of chemicals and API.
- Selection of suitable solvent.
- Selection of suitable wavelength.
- Development of UPLC method for the quantification of paroxetine and clonazepam in bulk and pharmaceutical dosage form.
- Validation of proposed method as per ICH Q2 (R1) guidelines.
- Estimate the amount of paroxetine and clonazepam bulk and pharmaceutical dosage form.

LITERATURE REVIEW

Nitasha Agrawall, 2013 A reliable and sensitive high performance liquid chromatographic method for the determination of widely prescribed antidepressant has been developed. Paroxetine is a potent selective serotonin reuptake inhibitor used for the treatment of depression and various mood disorders. The optimum mobile phase was prepared using a combination of 40% acetonitrile and 60% phosphate buffer 0.01 M to pH 3 and running under isocratic mode at a flow rate of 1.0 ml/min. with detection at 254nm. The applicability of the developed method is in the field of quality control as well as for monitoring the level of drug at various concentrations during synthesis of the parent drug. The suggested methodology was validated following the guidelines of the FDA in terms of: sensitivity (LOD and LOQ, 0.005 and 0.01 nag/mL, respectively), linearity between 0.5 - 50 nag/mL (r2 > 0.9999), inter- and intraday precision (< 0.259% and < 0.538%), robustness (less than 5.0%) and recovery (99.7 - 100.7%). The developed method is specific, rapid (less than 10 min), precise, reliable, accurate, cheap and suitable for routine analysis for the determination of paroxetine in pharmaceutical preparations.

Chusena Narasimharaju Bhimanadhuni, 2012, A Simple, efficient and reproducible reverse phase high performance liquid chromatographic method was developed and validated for the Simultaneous determination of paroxetine and Clonazepam in combined dosage form. The separation was effected on a Hypersil ODS C18 column (250mm X 4.6mm; 5µ) using a mobile phase mixture of buffer and acetonitrile in a ratio of 50:50 v/v at a flow rate of 1.0ml/min. The detection was made at 240nm. The retention time of paroxetine and Clonazepam was found to be 2.840± 0.007min and 4.007±0.006 min.Calibration curve was

linear over the concentration range of 20-120µg/ml and 1-6µg/ml for paroxetineand Clonazepam. All the analytical validation parameters were determined and found in the limit as per ICH guidelines, which indicates the validity of the method. The developed method is also found to be precise, accurate, specific, robust and rapid for the simultaneous determination of paroxetine and Clonazepam in tablet dosage forms.

Bhagyasree T*, et.al., 2014 A simple, precise, rapid, specific and accurate reverse phase high performance liquid chromatography method was developed for simultaneous estimation of Paroxetine and Clonazepam in pharmaceutical dosage form. Chromatographic separation was performed on Agilent Eclipse XDB (C) (4.6mm x 150mm, 5m) column, with mobile phase comprising of mixture of buffer (pH7, adjusted with ammonium acetate), acetonitrile in the ratio of 82:18v/v, at the flow rate 0.8 ml/min. The detection was carried out at 265 nm. The retention times of paroxetine and clonazepam were found to be 2.36 and 3.14 minsrespectively with a run time of 5 mins, theoretical levels for paroxetine and clonazepam were 6753 and 4693 respectively, wit a resolution of 5.10. As per ICH guidelines the method was validated for linearity, accuracy, precision, limit of detection and limit of quantification, robustness and ruggedness. Linearity of paroxetine was found in the range of 100-300µg/mL and that for clonazepam was found to be 2-6 µg/mL.

Moinuddin R Syed, 2010 A simple and reproducible method was developed for assay of paroxetine in tablets. The excipients in the commercial tablet preparation did not interfere wi th the assay. Beer's law is obeyed in the range 2.0-10.0µg/ml at max 294 nm. The molar absorptivity was calculated. Six triplicate analyses of solutions containing six different

concentrations of the examined drug were carried out and gave a mean correlation coefficient 0.999. The proposed method was applied to the determination of the examined drug in market tablet and the results demonstrated that the method is equally accura te, precise and reproducible as the official methods.

Srinivas Reddy*, et.al., 2014 A novel stability indicating reversed-phase liquid chromatographic method has been developed and validated for simultaneous estimation of paroxetine and clonazepam in combined pharmaceutical dosage form. An Agilent zorbax sb-c18 (250mmx4.6mmx5 μm) column with the mobile phase containing 0.2 % Orthophosphoric acid and Methanol (60:40 v/v) was used. The flow rate was maintained at 0.8 ml/min, column temperature was 30°C and effluents were monitored by using a photodiode array detector at 270 nm. The retention times of paroxetine and clonazepam were found to be 3.478min and 3.964 min, respectively. Correlation co-efficient for paroxetine and clonazepam were found to be 0.99and 0.99, respectively. The proposed method was validated with respect to linearity, accuracy, precision, specificity, and robustness. Recovery of paroxetine and clonazepam in formulations was found to be in a range of 97-103% and 97-103% respectively. Paroxetine and clonazepam were also subjected to the stress conditions of oxidative, acid, base, hydrolytic, thermal and photolytic degradation. The degradation products were well resolved from and peak purity test results confirmed that paroxetine and clonazepam peaks were homogenous and pure in all stress samples, thus proving stability-indicating power of the method. Due to its simplicity, rapidness and high precision, this method can be applied for regular analysis.

Lakshmi Surekha G*, et.al., 2012 A simple, precise, rapid, and reproducible RP -HPLC method was developed and validated for the determination of Paroxetine in Pharmaceutical dosage form. Separation was achieved under optimized chromatographic condition on a PhenomenaxLunaC (ODS) column (150 X 4.6 mm i.d., particle size5m).The mobile phase consisted of Phosphate buffer at pH 6.0: Acetonitrile in the ratio 50: 50 v/v. An isocratic elution at a flow rate of 2 ml/ min at ambient temperature. The detection was carried out at 265 nm. Using Shimadzu UV-Visible detector SpD-10AVP.The retention time of Paroxetine is found to be 3.5min and the calibration curve was linear in the concentration range of 20–100mg/ ml (r- 0.9999).The limit of detection and the limit of quantification were found to be 0.6327 mg/ml and 1.963 mg/ml respectively. The amount of Paroxetine present in the formulation was found to be 99.55. The method was validated statistically using the SD, %RSD and SE and the values are found to be within the limits and the recovery studies were performed and the percentage recoveries was found to be 99.53± 0.6327 %. So, the proposed method was found to be simple, specific, linear, an drugged. Hence it can be applied for routine analysis of Paroxetine in the Pharmaceutical formulations**.**

Aziz Unnisa*, et.al., 2014 The aim of the present study is to develop RP-HPLC-PDA method for the simultaneous estimation of Clonazepam (CLP) and Paroxetine hydrochloride (PAR). The method uses a Agilent C18 reverse phase column (150 x 4.6mm, 5µm) with mobile phase consisting of 15mM Ammonium acetate : Methanol (40:60 v/v) at isocratic mode with an injection volume of 10µL and the eluents were monitored at 254nm.The retention times of CLP and PAR were 4.01 and 5.42 min respectively and showed a good linearity in the concentration range of 1-5 µg/mL for CLP and 25-125µg/mL for PAR with a good correlation coefficient for both the drugs. The validation parameters like specificity,

system suitability, linearity, LOD, LOQ, precision, robustness were all within the limits as per ICHguidelines. The proposed RP-HPLC-PDA method is specific, accurate, precise and economic and can be successfully applied for the simultaneous estimation of CLP and PAR in bulk and tablet dosage forms.

Geetharam Yanamadala*, et.al., 2014 The study describes development and subsequent validation of a stability indicating reverse-phase high-performance liquid chromatography method for the simultaneous estimation of Paroxetine hydrochloride and clonazepam in tablet dosage forms. A reversed-phase Kromasil ,C18, (150mm x 4.6 mm, particle size) column with mobile phase consisting of Acetonitrile and 0.1 % Orthophosphoric acid buffer 60:40 (v/v) was used. The flow rate was 1.2 mLmin-1 and effluents were monitored at 260 nm. The retention times (RT) of Paroxetine and clonazepam were found to be 3.46 min and 4.55 min, respectively. The method was validated in terms of linearity, range, specificity, accuracy, and precision, limit of detection (LOD) and limit of quantification (LOQ). The linearity for both the drugs was found in the range of 125-750 µg/ml and 2.5-15 µg/ml for Paroxetine and clonazepam. The % recoveries of Paroxetine hydrochloride and clonazepam were found to be 99.4 -100.6 and 98.1-101.0 respectively. The utility of the procedure is verified by its application to marketed formulations that were subjected to accelerated degradation studies. The method distinctly separated the drug and degradation products even in actual samples. The products formed in marketed tablet dosage forms are similar to those formed during stress studies.

Erk N*, et.al., 2003 The antidepressant agent paroxetine hydrochloride (POT) was studied by cyclic voltammetry (CV),differential pulse voltammetry (DPV) and Oster young square wave voltammetry (OSWV). A sensitive method is described for the determination of POT in its pure form and in human plasma. The linear relationship between concentration and peak current permits the quantification of POT by CV, DPVand OSWV in the concentration range of $2x\ 10^{-5}$-$8x10^{-4}$ M. Applicability to tablets and human plasma analysis has been illustrated. Furthermore, a HPLC method with diode array detection was developed. Linearity was established between $2x\ 10^{-7}$-$6x10^{-5}$ M for POT. The described methods were successfully employed with high degrees of precision and accuracy for the estimation of total drug content in human plasma and pharmaceutical dosage forms of POT.

Chakole RD*, et.al., 2012 This paper presents a RP-HPLC method for simultaneous estimation of paroxetine and clonazepam in pharmaceutical formulations. The process was carried out on a 250 × 4.6 mm, 5µ, C18 column. The flow rate was 1ml/min and eluent was monitored by absorbance at 248 nm using a mixture of Methanol and Buffer (pH 4.0) in the ratio of 90:10 (v/v). The retention times of paroxetineand Clonazepam was found to be 3.22 and 4.29 min respectively. Calibration plots were linear in the concentration range of 2.5-80 µg mL-1 and 0.125-4 µg mL-1 for paroxetine and clonazepam respectively. The total run time is 10 min. The proposed method was validated by testing its linearity, recovery, specificity, system suitability, precision (Intraday and intraday), robustness andLOD/LOQ values and it was successfully employed for the simultaneous estimation of paroxetine and clonazepam in pharmaceutical tablet formulations.

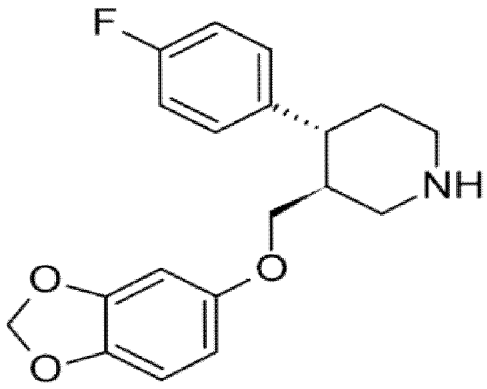
DRUG PROFILE

PAROXETINE

MOLECULAR FORMULA : $C_{19}\ H_{20}FNO_3$

MOLECULAR MASS:329.36

CHEMICAL STRUCTURE



MECHANISM OF ACTION

Paroxetine is a potent and highly selective inhibitor of neuronal serotonin reuptake. It likely inhibits the reuptake of serotonin at the neuronal membrane, enhances serotonergic neurotransmission by reducing turnover of the neurotransmitter, therefore it prolongs its activity at synaptic receptor sites and potentiates 5-HT in the CNS.

SOLUBILITY

It is freely soluble in methanol and ethanol, sparingly soluble in dichloro methane and slightly soluble in water

PHYSICAL STATE: Off white powder

IUPAC NAME:(3S,4R)-3-[(2H-1,3-benzodioxol-5yloxy)methyl]-4-(4- fluorophenyl)piper dine hydrochloride hemihydrates

INDICATION: Major depressive disorder (MDD), panic disorder with or without agoraphobia, obsessive-compulsive disorder (OCD), social anxiety disorder (social phobia), generalized anxiety disorder (GAD), post-traumatic stress disorder (PTSD), and premenstrual dysphoric disorder (PMDD).

PKA: 9.77

ADVERSE EFFECT : Constipation. Insomnia, blurred vision

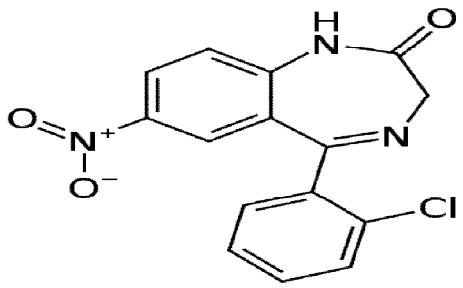
MELTING POINT:120 ℃ to 138 C

CLONAZEPAM

MOLECULAR FORMULA: $C_{15}H_{10}ClN_3O_3$

MOLECULAR MASS : 315.71

CHEMICAL STRUCTURE :



MECHANISM OF ACTION :

Allosteric interactions between central benzodiazepine receptors and gamma-amino butyric acid (GABA) receptors potentiate the effects of GABA. As GABA is an inhibitory neurotransmitter, this results in increased inhibition of the ascending reticular activating system. Benzodiazepines, in this way, block the cortical and limbic arousal that occurs following stimulation of the reticular pathways.

SOLUBILITY

It is practically insoluble in water, slightly soluble in alcohol and methanol and very slightly soluble in ether.

PHYSICAL STATE : Clonazepam is slightly yellowish, crystalline powder.

IUPAC NAME:5-(2-chlorophenyl)-7-nitro-2,3-dihydro-1H-1,4-benzodiazepin-2-one.

INDICATION:

Clonazepam is used as an anticonvulsant in the treatment of the Lennox-Gastautsyndrome (petit mal variant), alkinetic and myoclonic seizures. It can also be used for the treatment of panic disorders.

PKA:11.89

ADVERSE EFFECT : Drowsiness,Motor impairment, Confusion, Psychomotor agitation, Hallucinations.

MELTING POINT:238° C-240° C

MATERIALS AND METHODS

MATERIALS

CHEMICALS AND WORKING REFERENCE STANDARD

Paroxetine working reference standard -Sun pharma (Sikkim India)

Clonazepam working reference standard - Sun pharma (Sikkim India)

PottasiumDihydrogen orthophosphate -Merck limited(Mumbai)

Ortho phosphoric acid -Merck limited(Mumbai)

Acetonitrile HPLC –grade - M/S SD Fine chemicals (Mumbai)

Methanol HPLC –grade - M/S SD Fine chemicals (Mumbai)

Water HPLC-grade -Grade from Mille-QRO System.

EQUIPMENTS

Instrument/Equipment's used	Makers
UV-Visible spectrometer	Shimadzu UV-2550
UPLC	Thermo scientific
Sonicater	Remi instrument Ltd
Analytical balance	Metler Toledo-AG204
Detector	Photodiode Array Detector
pH Meter	Micropro Grademate-DI707
Analytical Column	Thermo Fischer scientific Hypercel C18 column (50x 2.1mm, 1.8µm).

METHODOLOGY

Method development

Selection of wavelength (λ_{max})

The sensitivity of the UPLC method that uses PDA detection depends upon the proper selection of the wavelength. An ideal wavelength is one that gives good response for the drugs to be detected. In a entire UV visible region both the drugs were strongly absorbed at 265nm. (Chromatogram no 1).So this wavelength was selected for further studies.

Trials for RP- UPLC method development

Trial – 1

Chromatographic conditions

UPLC	:	Thermo Scientific UPLC system.
Column	:	Thermo Fischer scientific Hypercel C18 column (50x 2.1mm, 1.8µm).
Flow Rate	:	0.5 ml/min
PDA Detection	:	265nm
Injection Volume	:	5µL
Temperature	:	25°C
Run Time	:	10 min.
Mobile phase	:	Acetonitrile: Water (70:30)

Diluent: Mobile phase.

Result:

Only one drug is eluted but failed in theoretical plates and tailing factor. (Chromatogram no. 5)

Trial – 2

Chromatographic conditions

UPLC	:	Thermo Scientific UPLC system.
Column	:	Thermo Fischer scientific Hypercel C18 column (50x 2.1mm, 1.8µm).
Flow Rate	:	0.5 ml/min
PDA Detection	:	265nm
Injection Volume	:	5µL
Temperature	:	25^{o}C
Run Time	:	10 min.
Mobile phase	:	Acetonitrile: methanol: Water(10:50:40)
Diluent	:	Mobile phase.

Result:

Both the peaks are eluted but failed in tailing factor. (Chromatogram no. 6).

Trial – 3

Chromatographic conditions

UPLC	:	Thermo Scientific UPLC system.
Column	:	Thermo Fischer scientific Hypercel C18 column (50x 2.1mm, 1.8µm).
Flow Rate	:	0.5 ml/min
PDA Detection	:	265nm
Injection Volume	:	5µL
Temperature	:	25^{o}C
Run Time	:	10 min.
Mobile phase	:	Acetonitrile: Methanol: Buffer (10:50:40)
Diluent	:	Mobile phase.

Result:

Paroxetine and Clonazepam are eluted at 1.285 and 2.458 respectively (Chromatogram no.7-12), efficiency parameters are indicate the good separation, asymmetric. So this method was selected for further analysis.

SIMULATION ESTIMATION OF PAROXETINE AND CLONAZEPAM

ASSAY:RP- UPLC METHOD

CHEMICAL AND WORKING REFERENCE STANDARD

- Paroxetine Working reference standard
- Clonazepam working reference standard
- Acetonitrile HPLC Grade
- Water HPLC Grade

TABLETS BRAND USED

PANZEP

REGENTS REQUIRED

272.19 mg of Potassium dihydrogen orthophosphate (2mM) was dissolved in sufficient water (HPLC grade) with aid ofsonicator and the volume was made up to 1000ml with water. Finally pH was adjusted to 3 with ortho phosphoric acid.

MOBILE PHASE:

Acetonitrile, Methanol and Buffer were mixed in the ratio of 10:50:40 and sonicated for 20minutes, Filtered with 0.45 µ membrane filter.

DILUENT: Mobile phase.

STANDARD SOLUTION :

SOLUTION (A)

Weighed accurately 50 mg paroxetine working referenestandard andtransferred carefully in to a100ml volumetric flask sufficient mobile phase and sonicated for 10 minutes ,cooled to room temperature and diluted makeup with 100ml volumetric flask.

SOLUTION (B)

Weighed accurately 10 mg clonazepam working referenestandard andtransferred carefully in to a100ml volumetric flask sufficient mobile phase and sonicated for 10 minutes ,cooled to room temperature and diluted makeup with 100ml volumetric flask.

MIXTURED STANDARD SOLUTION:

2 mL was pipetted out from the stock solution (B) and transferred in to a 100 mL volumetric flask,(Solution A) diluted up to the mark with mobile phase. Then the standard solution with the concentration of 500µg/mL of paroxetine and 20µg/mL of Clonazepam. Resulting solution was then filtered with 0.45 µ membrane filter.

PREPARATION OF SAMPLE SOLUTIONS

Twenty tablets were accurately weighed and finely powdered. A quantity of powder weight equivalent to 50mg of paroxetine and 2mg of clonazepam were weighed and transferred to a 100 mL volumetric flask and sufficient mobile phase was added to dissolve it. Then the solution was sonicated for 10 min. Final volume was adjusted with the mobile phase and filtered with 0.45 µ membrane filter. Then the sample solution with the concentration of 500µg/mL of paroxetine and 20µg/mL of Clonazepam. Resulting solution was then filtered with 0.45 µ membrane filter.

The amount of paroxetine and clonazepam present in each tablet were calculated by using the following formula:

Amount present

$$= \frac{\text{Sample Area}}{\text{Standard Area}} X \frac{\text{Standard weight}}{\text{Standard Dilution}} X \frac{\text{Sample Dil}}{\text{Sample weight}} X \text{Average weight}$$

$$\% \text{ Amount present} = \frac{\text{Amount present}}{\text{Label Claim}} X 100$$

RESULTS AND DISCUSSION

Validation of developed method

The developed method was validated according to ICH guidelines. The method was validated in terms of specificity, system suitability, linearity, precision, accuracy, robustness, LOD and LOQ.

Specificity

Specificity of the method was established by injecting the blank and placebo (synthetic mixtures). No interference was observed between the placebo and blank with principal peaks and hence the method was specific for these two drugs.

System suitability

System performance was determined by system suitability parameters such as retention time, theoretical plates,asymmetric factor and resolution were calculated and percentage RSD was found to be less than 2 % indicating good performance of the system (Chromatogram n7-12).

Linearity

Linearity of the method was established by analysis of mixed standard solution containing 250-750 µg/ml for Paroxetine and 10-30µg/ml for Clonazepam. The calibration curves drawn by plotting the response versus concentration were found to be linear and their coefficient of correlations (R^2) values are 0.9993 and 0.9997 for paroxetine and Clonazepam respectively(Table 1& 2), indication of good correlation between concentration and responses (Chromatogram no13-17).

Table 1 **Linearity for Paroxetine**

concentration	Sample area
250	215843
375	321456
500	433557
625	546824
750	643974

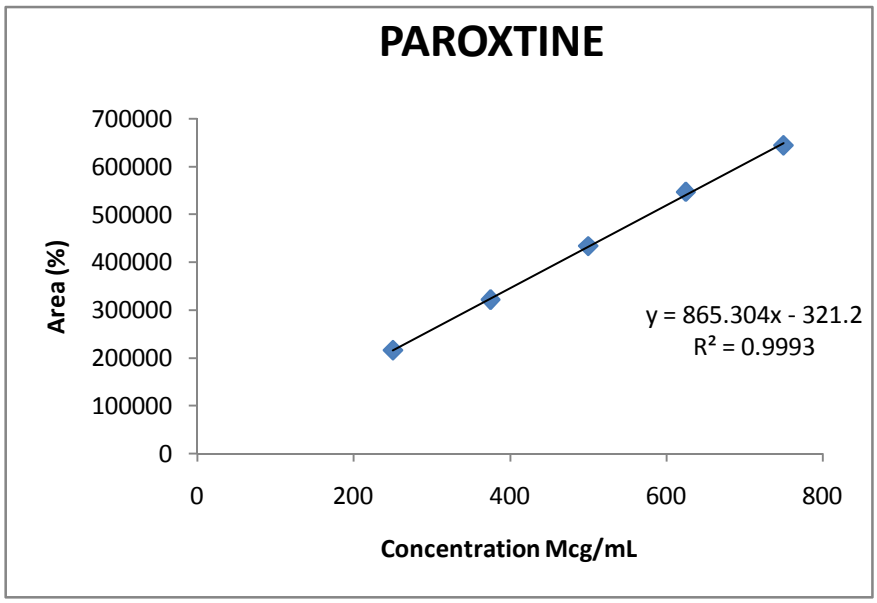
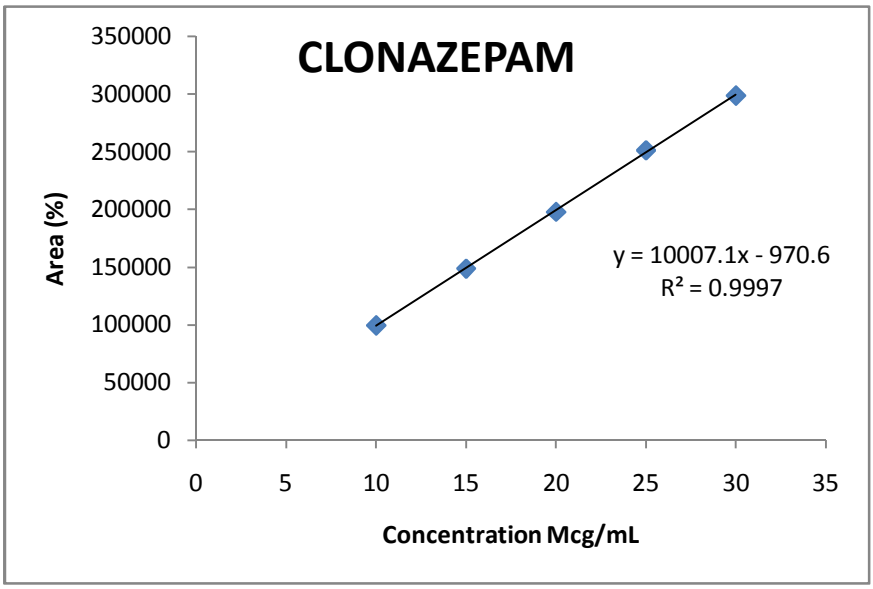


Table 2 **Linearity for Clonazepam**

concentration	Sample area
10	99571
15	148734
20	197855
25	251163
30	298534



Accuracy

Accuracy of the method was checked by recovery studies at the level of 50%, 100% and 150% of known amount of Paroxetine and clonazepam were added to the placebo from the label claim. Paroxetine and clonazepam recovered in all the levels were found to be close to 100%, indicates that the accuracy of the proposed method (Table 3-8), (Chromatogram no. 18- 26).

Table 3 Accuracy of Paroxetine 50 %

Sample area	Std area	Added amt	Amt Recovery	% Amt recovery
218362	433294	25	25.07191307	100.2877
219449	433294	25	25.19672036	100.7869
221846	433294	25	25.47193938	101.8878
		AVG	25.2468576	100.9874
		SD	0.204671858	0.818687
		% RSD	0.810682507	0.810683

Table 4 Accuracy of Paroxetine 100 %

sample area	Std area	Added amt	Amt Recovery	% Amt recovery
433621	433294	50	49.78754552	99.57509
433527	433294	50	49.77675262	99.55351
433612	433294	50	49.78651216	99.57302
		AVG	49.78360343	99.56721
		SD	0.005955435	0.011911
		% RSD	0.011962643	0.011963

Table 5 Accuracy of Paroxetine 150 %

Sample area	Std area	Added amt	Amt Recovery	% Amt recovery
653457	433294	75	75.02870049	100.0383
657891	433294	75	75.53780401	100.7171
657487	433294	75	75.49141749	100.6552
		AVG	75.35264066	100.4702
		SD	0.28149752	0.37533
		% RSD	0.373573531	0.373574

Table 6 Accuracy of Clonazepam 50 %

Sample area	**Std area**	**Added amt**	**Amt Recovery**	**% Amt recovery**
99612	199268	5	4.958905	99.1781
99214	199268	5	4.939091	98.78183
99254	199268	5	4.941083	98.82166
		AVG	4.94636	98.92719
		SD	0.01091	0.218198
		% RSD	0.220565	0.220565

Table 7 Accuracy of Clonazepam100 %

sample area	**Std area**	**Added amt**	**Amt Recovery**	**% Amt recovery**
199214	199268	10	9.917312	99.17312
199351	199268	10	9.924132	99.24132
199345	199268	10	9.923833	99.23833
		AVG	9.921759	99.21759
		SD	0.003854	0.038543
		%RSD	0.038847	0.038847

Table 8 Accuracy of Clonazepam150 %

Sample area	Std area	Added amt	Amt Recovery	% Amt recovery
298457	199268	15	14.85785	99.05231
298075	199268	15	14.83883	98.92553
297458	199268	15	14.80811	98.72076
		AVG	14.83493	98.89954
		SD	0.025094	0.167297
		% RSD	0.169158	0.169158

Precision

Precision study was established by injecting the sample solution (multiple sampling of the same homogeneous sample)without changing the assay procedure and the results were presented in table 9-32. Precision may be considered at three levels: repeatability, intermediate precision and reproducibility

Repeatability

This study was performed with a minimum of six replicate measurements of sample solution at a same day (Chromatogram no. 27-32).

Intermediate Precision

Intermediate precision was performed by injecting the sample solution in three different days, by different analysts and in different instruments(Chromatogram no. 33-41).

Reproducibility

Reproducibility of the method was checked in two laboratories and the results were compared.(Chromatogram no. 42-43).

The low % RSD (< 2 %) for Paroxetine and clonazepam indicated that the method is precise.

Table 9 Evaluation of precision study [paroxetine 0hrs]

Sample area	Std area	Avgwt	Amt present	% Amt present
431257	433294	262.9	12.37902888	99.03223
435784	433294	262.9	12.50897428	100.0718
432175	433294	262.9	12.40537963	99.24304
435784	433294	262.9	12.50897428	100.0718
431278	433294	262.9	12.37963167	99.03705
432587	433294	262.9	12.4172059	99.33765
		AVG	12.4331991	99.46559
		SD	0.060526594	0.484213
		% RSD	0.486814319	0.486814

Table 10 Evaluation of precision study [paroxetine 8hrs]

Sample area	Std area	Avgwt	Amt present	% Amt present
431257	433294	262.9	12.37902888	99.03223
432157	433294	262.9	12.40486295	99.2389
439815	433294	262.9	12.62468223	100.9975
433756	433294	262.9	12.45076149	99.60609
432150	433294	262.9	12.40466202	99.2373
432517	433294	262.9	12.41519658	99.32157
		AVG	12.44653236	99.57226
		SD	0.090314316	0.722515
		% RSD	0.725618298	0.725618

Table 11 Evaluation of precision study [paroxetine 16hrs]

sample area	Std area	AVG wt	Amt present	% Amt present
432165	433294	262.9	12.40509259	99.24074
432157	433294	262.9	12.40486295	99.2389
436844	433294	262.9	12.53940108	100.3152
432171	433294	262.9	12.40526481	99.24212
435274	433294	262.9	12.49433497	99.95468
432457	433294	262.9	12.41347431	99.30779
		AVG	12.44373845	99.54991
		SD	0.05850178	0.468014
		% RSD	0.470130264	0.47013

Table 12 Evaluation of precision study [Clonazepam 0hrs]

Sample area	Std area	AVG wt	Amt present	% Amt present
199872	199268	262.9	0.497503	99.50068
199147	199268	262.9	0.495699	99.13976
199247	199268	262.9	0.495948	99.18955
199254	199268	262.9	0.495965	99.19303
199578	199268	262.9	0.496772	99.35432
199587	199268	262.9	0.496794	99.35881
		AVG	0.496447	99.28936
		SD	0.00069	0.138074
		% RSD	0.139062	0.139062

Table 13 Evaluation of precision study [Clonazepam 8hrs]

Sample area	Std area	AVG wt	Amt present	% Amt present
199874	199268	262.9	0.497508	99.50168
199324	199268	262.9	0.496139	99.22788
199265	199268	262.9	0.495993	99.19851
199872	199268	262.9	0.497503	99.50068
199771	199268	262.9	0.497252	99.4504
199358	199268	262.9	0.496224	99.2448
		AVG	0.49677	99.35399
		SD	0.000723	0.144657
		% RSD	0.145598	0.145598

Table 14 Evaluation of precision study [Clonazepam 16hrs]

sample area	Std area	AVG wt	Amt present	% Amt present
199257	199268	262.9	0.495973	99.19452
199876	199268	262.9	0.497513	99.50268
198733	199268	262.9	0.494668	98.93367
198001	199268	262.9	0.492846	98.56926
198745	199268	262.9	0.494698	98.93964
199325	199268	262.9	0.496142	99.22838
		AVG	0.495307	99.06136
		SD	0.001603	0.320522
		% RSD	0.323559	0.323559

Table 15 Evaluation of precision study [paroxetine Day 1]

Sample area	Std area	AVG wt	Amt present	% Amt present
435781	433294	262.9	12.50888816	100.0711
432105	433294	262.9	12.40337032	99.22696
430178	433294	262.9	12.34805669	98.78445
432517	433294	262.9	12.41519658	99.32157
432014	433294	262.9	12.4007582	99.20607
432178	433294	262.9	12.40546575	99.24373
		AVG	12.41362262	99.30898
		SD	0.052371805	0.418974
		% RSD	0.421889779	0.42189

Table 16 Evaluation of precision study [Clonazepam Day 1]

Sample area	Std area	AVG wt	Amt present	% Amt present
199875	199268	262.9	0.497511	99.50218
198756	199268	262.9	0.494726	98.94512
197546	199268	262.9	0.491714	98.34275
198763	199268	262.9	0.494743	98.9486
199782	199268	262.9	0.497279	99.45588
199763	199268	262.9	0.497232	99.44642
		AVG	0.495534	99.10682
		SD	0.002268	0.453534
		% RSD	0.457621	0.457621

Table 17 Evaluation of precision study [paroxetine Day 2]

sample area	Std area	AVG wt	Amt present	% Amt present
432517	433294	262.9	12.41519658	99.32157
430214	433294	262.9	12.34909005	98.79272
432104	433294	262.9	12.40334161	99.22673
432017	433294	262.9	12.40084432	99.20675
432107	433294	262.9	12.40342772	99.22742
432117	433294	262.9	12.40371477	99.22972
		Avg	12.39593584	99.16749
		SD	0.023499288	0.187994
		% RSD	0.18957252	0.189573

Table 18 Evaluation of precision study [Clonazepam Day 2]

Sample area	Std area	AVG wt	Amt present	% Amt present
198075	199268	262.9	0.49303	98.6061
199874	199268	262.9	0.497508	99.50168
199354	199268	262.9	0.496214	99.24281
199842	199268	262.9	0.497429	99.48575
198677	199268	262.9	0.494529	98.90579
198768	199268	262.9	0.494755	98.95109
		Avg	0.495578	99.11554
		SD	0.001779	0.355789
		% RSD	0.358964	0.358964

Table 19 Evaluation of precision study [paroxetine Day 3]

sample area	Std area	Avg. Wt	Amt present	% Amt present
435102	433294	262.9	12.48939779	99.91518
435012	433294	262.9	12.48681438	99.89452
431187	433294	262.9	12.37701956	99.01616
435551	433294	262.9	12.50228612	100.0183
438452	433294	262.9	12.58555796	100.6845
435780	433294	262.9	12.50885946	100.0709
		AVG	12.49165588	99.93325
		SD	0.066927708	0.535422
		% RSD	0.535779316	0.535779

Table 20 Evaluation of precision study [Clonazepam Day 3]

Sample area	Std area	Avg.wt	Amt present	% Amt present
199324	199268	262.9	0.496139	99.22788
198752	199268	262.9	0.494716	98.94312
198754	199268	262.9	0.494721	98.94412
198764	199268	262.9	0.494745	98.9491
198757	199268	262.9	0.494728	98.94561
198756	199268	262.9	0.494726	98.94512
		AVG	0.494962	98.99249
		SD	0.000577	0.115333
		% RSD	0.116507	0.116507

Table 21 Evaluation of precision study [paroxetine Instrument 1]

sample area	Std area	Avgwt	Amt present	% Amt present
432157	433294	262.9	12.40486295	99.2389
431447	433294	262.9	12.38448274	99.07586
432718	433294	262.9	12.42096619	99.36773
432571	433294	262.9	12.41674663	99.33397
435781	433294	262.9	12.50888816	100.0711
435178	433294	262.9	12.49157933	99.93263
		AVG	12.437921	99.50337
		SD	0.050199317	0.401595
		% RSD	0.403598936	0.403599

Table 22 Evaluation of precision study [Clonazepam Instrument 1]

Sample area	Std area	Avgwt	Amt present	% Amt present
199875	199268	262.9	0.497511	99.50218
199524	199268	262.9	0.496637	99.32744
198754	199268	262.9	0.494721	98.94412
199436	199268	262.9	0.496418	99.28363
198767	199268	262.9	0.494753	98.95059
196874	199268	262.9	0.490041	98.00821
		AVG	0.495013	99.0027
		SD	0.002673	0.534604
		% RSD	0.53999	0.53999

Table 23 Evaluation of precision study [paroxetine Instrument 2]

Sample area	Std area	Avgwt	Amt present	% Amt present
435784	433294	262.9	12.50897428	100.0718
436641	433294	262.9	12.53357406	100.2686
438001	433294	262.9	12.57261222	100.5809
436800	433294	262.9	12.53813808	100.3051
432172	433294	262.9	12.40529352	99.24235
435471	433294	262.9	12.49998976	99.99992
		AVG	12.50976365	100.0781
		SD	0.057152684	0.457221
		% RSD	0.456864615	0.456865

Table 24 Evaluation of precision study [Clonazepam Instrument 2]

sample area	Std area	Avg. wt	Amt present	% Amt present
198687	199268	262.9	0.494554	98.91077
198756	199268	262.9	0.494726	98.94512
198754	199268	262.9	0.494721	98.94412
198751	199268	262.9	0.494713	98.94263
199358	199268	262.9	0.496224	99.2448
199357	199268	262.9	0.496222	99.24431
		AVG	0.495193	99.03862
		SD	0.0008	0.160034
		% RSD	0.161587	0.161587

Table 25 Evaluation of precision study [paroxetine Analyst 1]

sample area	Std area	AVG wt	Amt present	% Amt present
432541	433294	262.9	12.41588549	99.32708
432517	433294	262.9	12.41519658	99.32157
430012	433294	262.9	12.34329174	98.74633
432157	433294	262.9	12.40486295	99.2389
436687	433294	262.9	12.53489447	100.2792
436514	433294	262.9	12.52992858	100.2394
		AVG	12.44067663	99.52541
		SD	0.075991028	0.607928
		%RSD	0.610827132	0.610827

Table 26 Evaluation of precision study [Clonazepam Analyst 1]

sample area	Std area	Avgwt	Amt present	% Amt present
199687	199268	262.9	0.497043	99.40859
199344	199268	262.9	0.496189	99.23783
199752	199268	262.9	0.497205	99.44095
199762	199268	262.9	0.49723	99.44592
199325	199268	262.9	0.496142	99.22838
196582	199268	262.9	0.489314	97.86285
		AVG	0.49552	99.10409
		SD	0.00308	0.615963
		% RSD	0.621531	0.621531

Table 27 Evaluation of precision study [paroxetine Analyst 2]

sample area	Std area	AVG wt	Amt present	% Amt present
432157	433294	262.9	12.40486295	99.2389
432510	433294	262.9	12.41499565	99.31997
436571	433294	262.9	12.53156474	100.2525
435781	433294	262.9	12.50888816	100.0711
432578	433294	262.9	12.41694756	99.33558
436871	433294	262.9	12.5401761	100.3214
		AVG	12.46957253	99.75658
		SD	0.063732206	0.509858
		% RSD	0.511101768	0.511102

Table 28 Evaluation of precision study [Clonazepam Analyst 2]

sample area	Std area	Avgwt	Amt present	% Amt present
198756	199268	262.9	0.494726	98.94512
198976	199268	262.9	0.495273	99.05464
198750	199268	262.9	0.494711	98.94213
199024	199268	262.9	0.495393	99.07853
199024	199268	262.9	0.495393	99.07853
199875	199268	262.9	0.497511	99.50218
		AVG	0.495501	99.10019
		SD	0.001034	0.206707
		% RSD	0.208584	0.208584

Table 29 Evaluation of precision study paroxetine Lab 1]

sam area	Std area	AVG wt	Amt present	% Amt present
430217	433294	262.9	12.34917617	98.79341
435879	433294	262.9	12.51170121	100.0936
435712	433294	262.9	12.50690755	100.0553
432570	433294	262.9	12.41671792	99.33374
432470	433294	262.9	12.41384747	99.31078
432201	433294	262.9	12.40612595	99.24901
		AVG	12.43407938	99.47264
		SD	0.063292639	0.506341
		% RSD	0.509025535	0.509026

Table 30 Evaluation of precision study [Clonazepam Lab 1]

sample area	**Std area**	**AVG wt**	**Amt present**	**% Amt present**
199870	199268	262.9	0.497498	99.49969
199400	199268	262.9	0.496329	99.26571
199330	199268	262.9	0.496154	99.23086
199647	199268	262.9	0.496943	99.38867
199876	199268	262.9	0.497513	99.50268
199235	199268	262.9	0.495918	99.18357
		AVG	0.496726	99.3452
		SD	0.000693	0.138614
		% RSD	0.139528	0.139528

Table 31 Evaluation of precision study [paroxetine Lab 2]

Sample area	Std area	AVG wt	Amt present	% Amt present
433628	433294	262.9	12.44708731	99.5767
433256	433294	262.9	12.43640923	99.49127
433158	433294	262.9	12.43359618	99.46877
433621	433294	262.9	12.44688638	99.57509
433281	433294	262.9	12.43712684	99.49701
433694	433294	262.9	12.44898181	99.59185
		AVG	12.44168129	99.53345
		SD	0.006686139	0.053489
		% RSD	0.053739831	0.05374

Table 32 Evaluation of precision study [Clonazepam Lab 2]

sample area	Std area	AVG wt	Amt present	% Amt present
199025	199268	262.9	0.495395	99.07903
199878	199268	262.9	0.497518	99.50367
199644	199268	262.9	0.496936	99.38718
199847	199268	262.9	0.497441	99.48824
199735	199268	262.9	0.497162	99.43248
199358	199268	262.9	0.496224	99.2448
		AVG	0.49678	99.3559
		SD	0.000822	0.164434
		% RSD	0.1655	0.1655

Robustness

Robustness of the method was checked by small deliberate changes in the method parameters such as wavelength (±2nm), flow rate (±0.2ml), mobile phase ratio (±2%) and pH (± 0.05) but this changes, not affected the method results (Table 44-48), (Chromatogram no. 44-51).

The % RSD value calculated from the robustness study was found to be less than 2 % for Paroxetine and clonazepam, indicated that the method is robust.

Table 33 By changing the wavelength + 1nm of paroxetine

Sample area	Std area	Avgwt	Amt present	% Amt present
433625	433294	262.9	12.4470012	99.57601
433158	433294	262.9	12.43359618	99.46877
433691	433294	262.9	12.4488957	99.59117
		AVG	12.44316436	99.54531
		SD	0.00834025	0.066722
		% RSD	0.067026765	0.067027

Table 34 By changing the wavelength + 1nm of Clonazepam

Sample area	Std area	Avgwt	Amt present	% Amt present
199865	199268	262.9	0.497486	99.4972
199766	199268	262.9	0.49724	99.44792
199872	199268	262.9	0.497503	99.50068
		AVG	0.49741	99.48193
		SD	0.000148	0.029512
		% RSD	0.029665	0.029665

Table 35 By changing the wavelength - 1nm of paroxetine

Sample area	**Std area**	**Avgwt**	**Amt present**	**% Amt present**
433261	433294	262.9	12.43655275	99.49242
433157	433294	262.9	12.43356748	99.46854
433822	433294	262.9	12.45265599	99.62125
		AVG	12.44092541	99.5274
		SD	0.010268053	0.082144
		% RSD	0.082534479	0.082534

Table 36 By changing the wavelength - 1nm of Clonazepam

Sample area	Std area	avgwt	Amt present	% Amt present
199785	199268	262.9	0.497287	99.45737
199874	199268	262.9	0.497508	99.50168
199586	199268	262.9	0.496792	99.35831
		AVG	0.497196	99.43912
		SD	0.000367	0.073409
		% RSD	0.073823	0.073823

Table 37 By changing the flow rate +10µl of paroxetine

sample area	Std area	Avgwt	Amt present	% Amt present
433871	433294	262.9	12.45406251	99.6325
433697	433294	262.9	12.44906793	99.59254
433965	433294	262.9	12.45676074	99.65409
		AVG	12.45329706	99.62638
		SD	0.003903112	0.031225
		%RSD	0.031341999	0.031342

Table 38 By changing the flow rate +10µl of Clonazepam

Sample area	Std area	Avgwt	Amt present	% Amt present
199876	199268	262.9	0.497513	99.50268
199832	199268	262.9	0.497404	99.48077
199887	199268	262.9	0.497541	99.50815
		AVG	0.497486	99.4972
		SD	7.24E-05	0.014488
		% RSD	0.014561	0.014561

Table 39 By changing the flow rate -10µl of paroxetine

Sample area	Std area	Avgwt	Amt present	% Amt present
433658	433294	262.9	12.44794845	99.58359
432315	433294	262.9	12.40939827	99.27519
433382	433294	262.9	12.440026	99.52021
		AVG	12.43245757	99.45966
		SD	0.020359026	0.162872
		% RSD	0.163757054	0.163757

Table 40 By changing the flow rate -10µl of Clonazepam

Sample area	Std area	Avgwt	Amt present	% Amt present
199005	199268	262.9	0.495345	99.06907
199024	199268	262.9	0.495393	99.07853
199324	199268	262.9	0.496139	99.22788
		AVG	0.495626	99.12516
		SD	0.000445	0.089081
		% RSD	0.089868	0.089868

Table 41 By changing pH + 0.05 of Paroxetine

Sample area	Std area	Avgwt	Amt present	% Amt present
433258	433294	262.9	12.43646664	99.49173
433197	433294	262.9	12.43471566	99.47773
433175	433294	262.9	12.43408416	99.47267
		AVG	12.43508882	99.48071
		SD	0.001234295	0.009874
		% RSD	0.009925902	0.009926

Table 42 By changing pH + 0.05 of Clonazepam

Sample area	Std area	Avgwt	Amt present	% Amt present
199365	199268	262.9	0.496241	99.24829
199866	199268	262.9	0.497488	99.4977
199357	199268	262.sss9	0.496222	99.24431
		AVG	0.49665	99.3301
		SD	0.000726	0.14516
		% RSD	0.146139	0.146139

Table 43 By changing pH - 0.05 of paroxetine

sam area	Std area	Avgwt	Amt present	% Amt present
433256	433294	262.9	12.43640923	99.49127
433186	433294	262.9	12.43439991	99.4752
433475	433294	262.9	12.44269552	99.54156
		AVG	12.43783489	99.50268
		SD	0.004327662	0.034621
		% RSD	0.034794334	0.034794

Table 44 By changing pH - 0.05 of Clonazepam

sample area	Std area	AVG wt	Amt present	% Amt present
199650	199268	262.9	0.496951	99.39017
199057	199268	262.9	0.495475	99.09496
198862	199268	262.9	0.494989	98.99788
		AVG	0.495805	99.161
		SD	0.001022	0.204311
		%RSD	0.20604	0.20604

Table 45 By changing mobile phase + 2% of paroxetine

Sample area	Std area	AVG wt.	Amt present	% Amt present
432157	433294	262.9	12.40486295	99.2389
435781	433294	262.9	12.50888816	100.0711
435175	433294	262.9	12.49149322	99.93195
		AVG	12.46841478	99.74732
		SD	0.055720481	0.445764
		% RSD	0.446893064	0.446893

Table 46 By changing mobile phase + 2% of Clonazepam

Sample area	Std area	AVG wt	Amt present	% Amt present
199802	199268	262.9	0.497329	99.46584
199346	199268	262.9	0.496194	99.23883
199863	199268	262.9	0.497481	99.4962
		AVG	0.497001	99.40029
		SD	0.000703	0.140651
		% RSD	0.141499	0.141499

Table 47 By changing mobile phase - 2% of paroxetine

Sample area	**Std area**	**Avgwt**	**Amt present**	**% Amt present**
432157	433294	262.9	12.40486295	99.2389
432578	433294	262.9	12.41694756	99.33558
435171	433294	262.9	12.4913784	99.93103
		AVG	12.43772964	99.50184
		SD	0.046852447	0.37482
		% RSD	0.37669614	0.376696

Table 48 By changing mobile phase - 2% of Clonazepam

Sample area	Std area	Avgwt	Amt present	% Amt present
198725	199268	262.9	0.494648	98.92968
199025	199268	262.9	0.495395	99.07903
198738	199268	262.9	0.494681	98.93615
		AVG	0.494908	98.98162
		SD	0.000422	0.084419
		% RSD	0.085288	0.085288

Limit of Detection (LOD) and Limit of Quantification (LOQ*)*

LOD and LOQ were calculated based on the standard deviation of the response (50% concentration solution) and the slope of calibration graph. LOD and LOQ were found to be 138.36 ng/ml and 419.29ng/ml for Paroxetine 74.81ng/ml and 226.72ng/ml for Clonazepam.

Stability

Standard and sample Solutions stability were checked up to 3 days at room temperature and the Reponses was measured on one time at each day. Results revealed that, there was no degradation of Paroxetine and clonazepam during this period. The results were presented in Table 49-50.

Table 49 Evaluation of Stability studies of paroxetine

Sample area	Std area	Avgwt	Amt present	% Amt present
432157	433294	262.9	12.40486295	99.2389
435712	433294	262.9	12.50690755	100.0553
432178	433294	262.9	12.40546575	99.24373

Table 50 Evaluation of Stability studies of Clonazepam

sample area	Std area	Avgwt	Amt present	% Amt present
199876	199268	262.9	0.497513	99.50268
199873	199268	262.9	0.497506	99.50118
199876	199268	262.9	0.497513	99.50268

All the validation parameters results revealed that, the developed and optimized method was suitable, linear, precise, accurate and robust for the simultaneous estimation of Paroxetineand clonazepam in pharmaceutical dosage form

Method application to the marketed dosage form

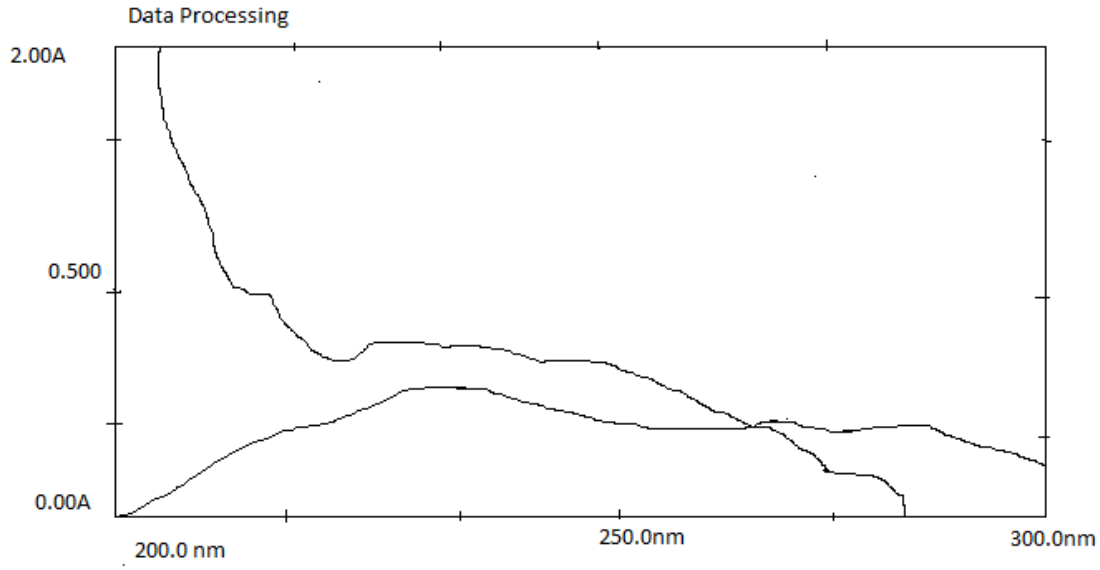
Assay was performed on marketed dosage form as per the above described procedure. Six replicate injections of sample solutions were given in to HPLC system without changing the proposed method conditions and the amount Paroxetine and clonazepam present in each tablet was calculated and found to be 501.64mg and 403.54mg respectively (Table 51), (Chromatogram no. 52)

Table 51 Assay results for commercial formulation

Paroxetine		Clonazepam	
Amount present (mg)	Percentage (%)	Amount present (mg)	Percentage (%)
12.47	99.76	0.501	100.00
12.52	100.00	0.492	98.40
12.44	99.52	0.489	97.80
12.43	99.44	0.489	97.80
12.49	99.92	0.494	98.80
12.54	100.32	0.499	99.80

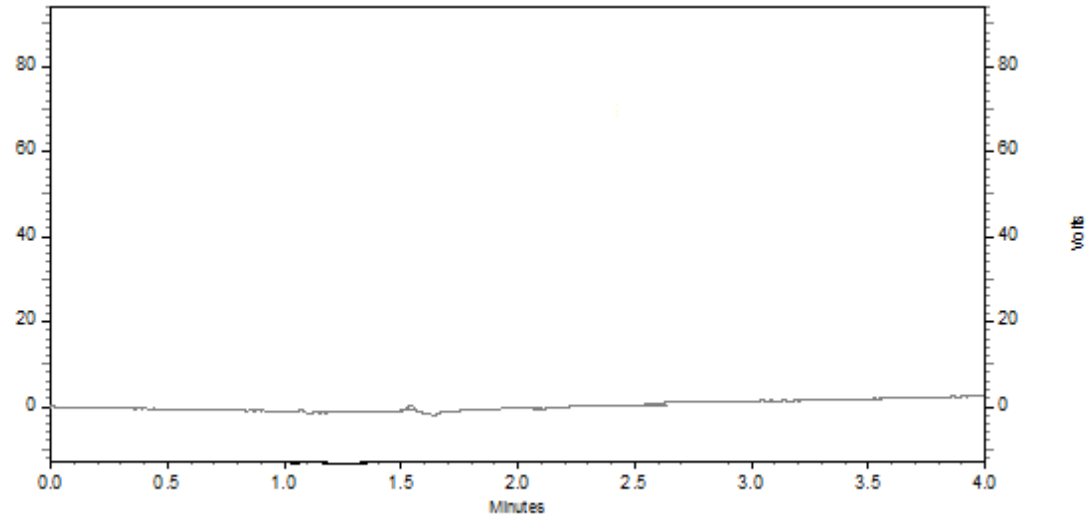
CHROMATOGRAM-1

Selection of wavelength



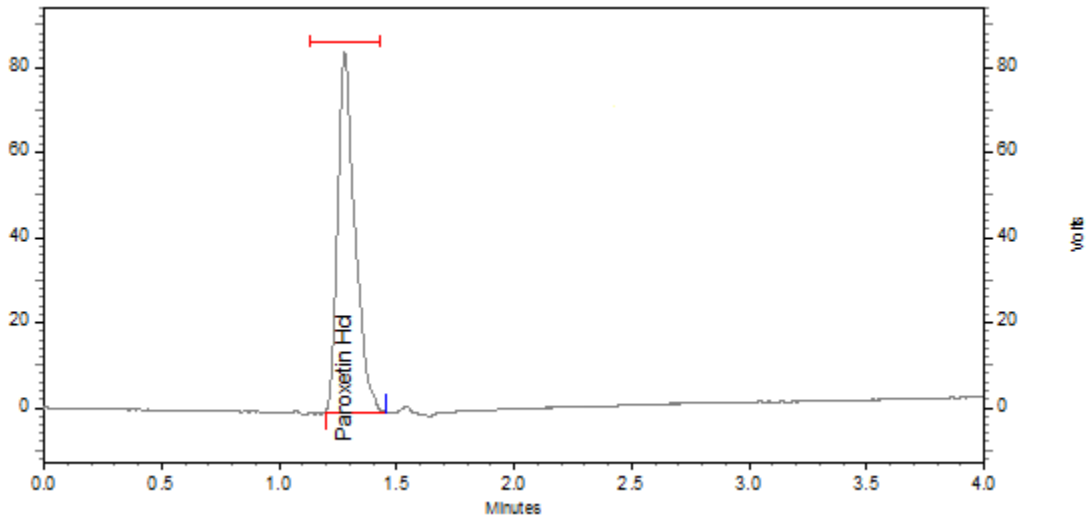
CHROMATOGRAM-2

MOBILE PHASE



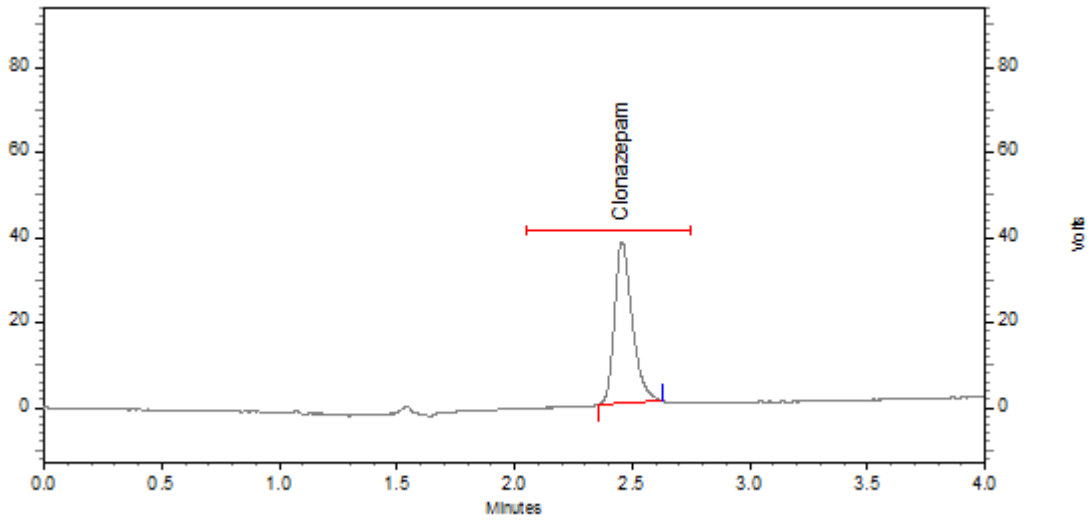
CHROMATOGRAM-3

REFERENCE STANDARD FOR PAROXETINE



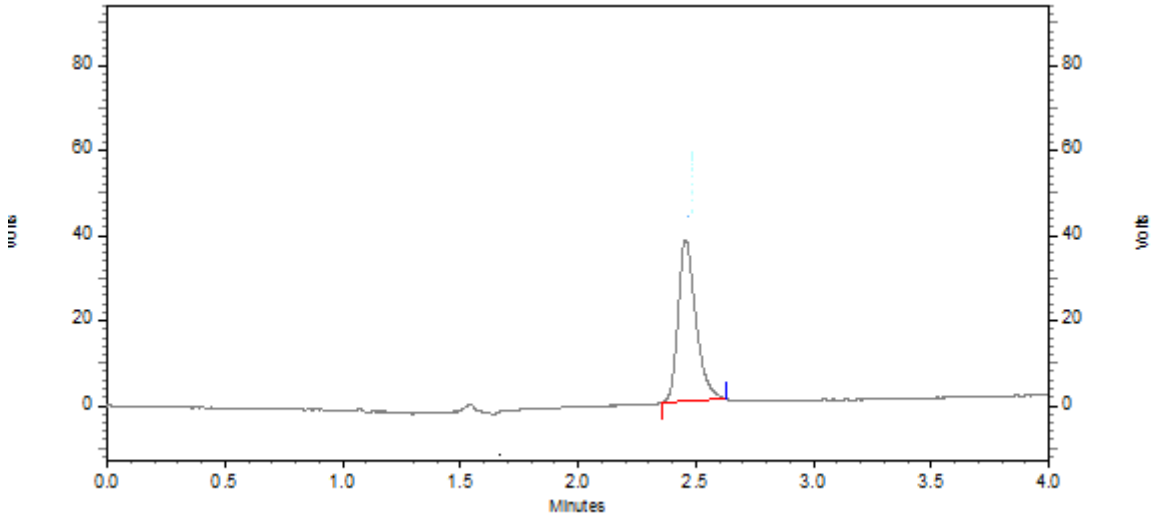
CHROMATOGRAM-4

REFERENCE STANDARD FOR CLONAZEPAM

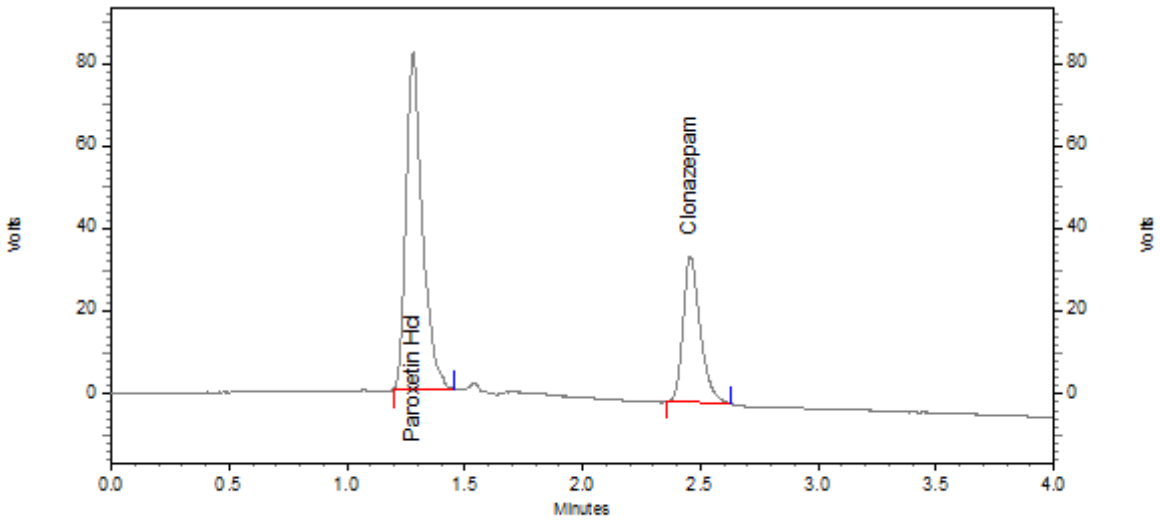


CHROMATOGRAM-5

TRAIL-1

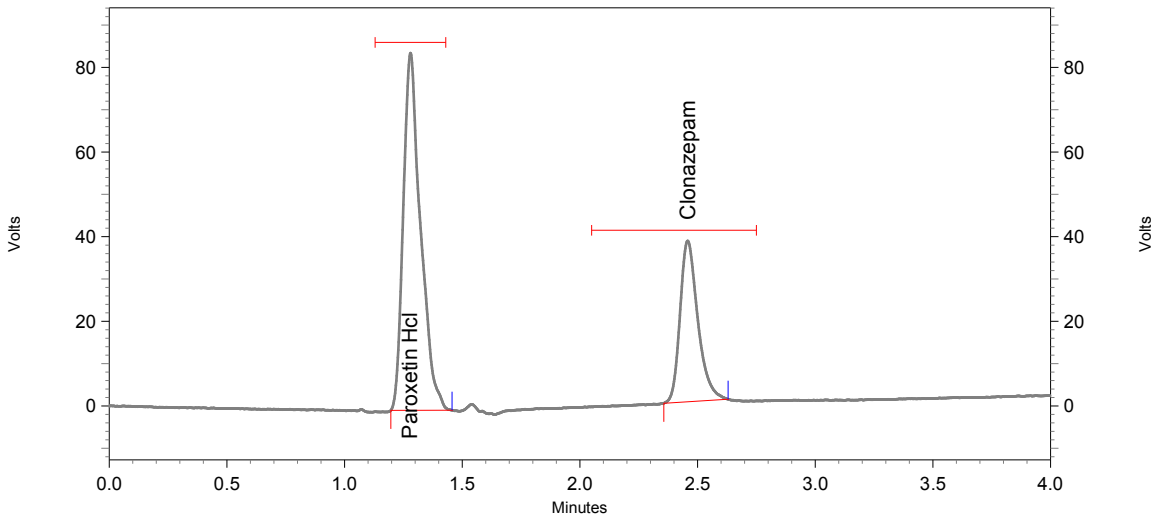


CHROMATOGRAM-6
TRAIL-2

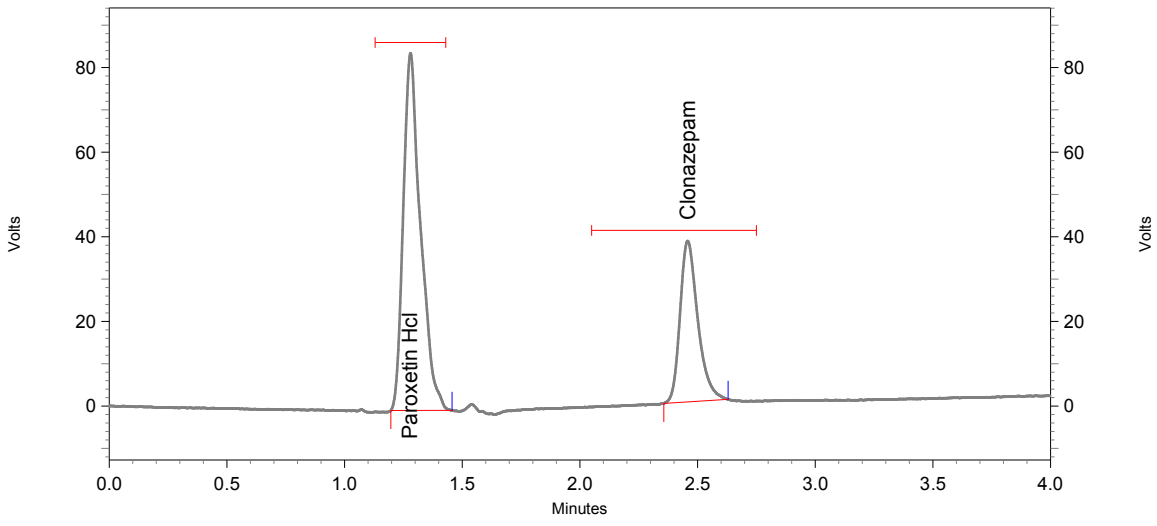


SYSTEM SUITABILITY

CHROMATOGRAM NO-7
System suitability 1

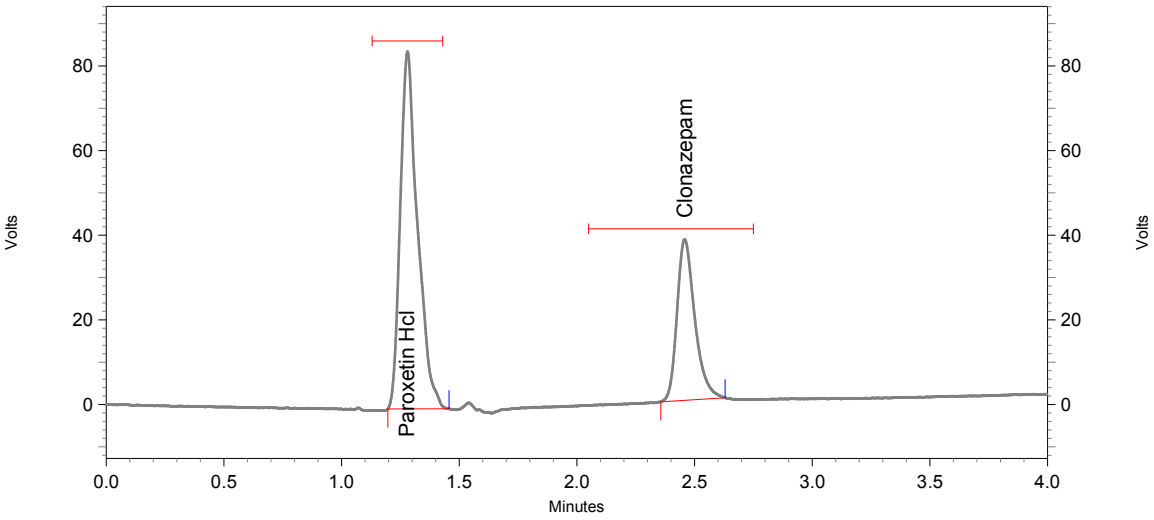


CHROMATOGRAM NO -8
System suitability 2



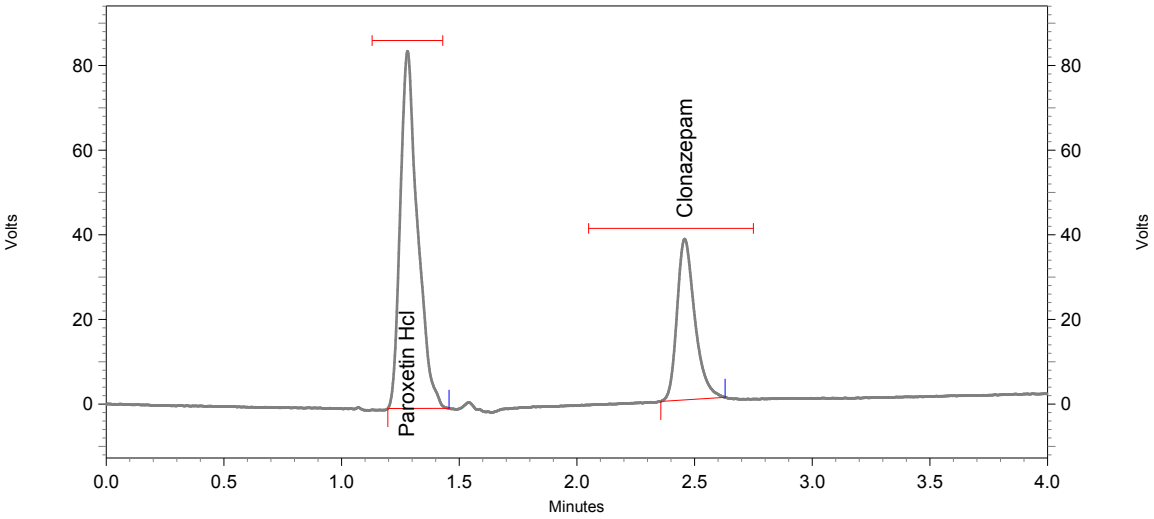
CHROMATOGRAM NO -9

System suitability 3



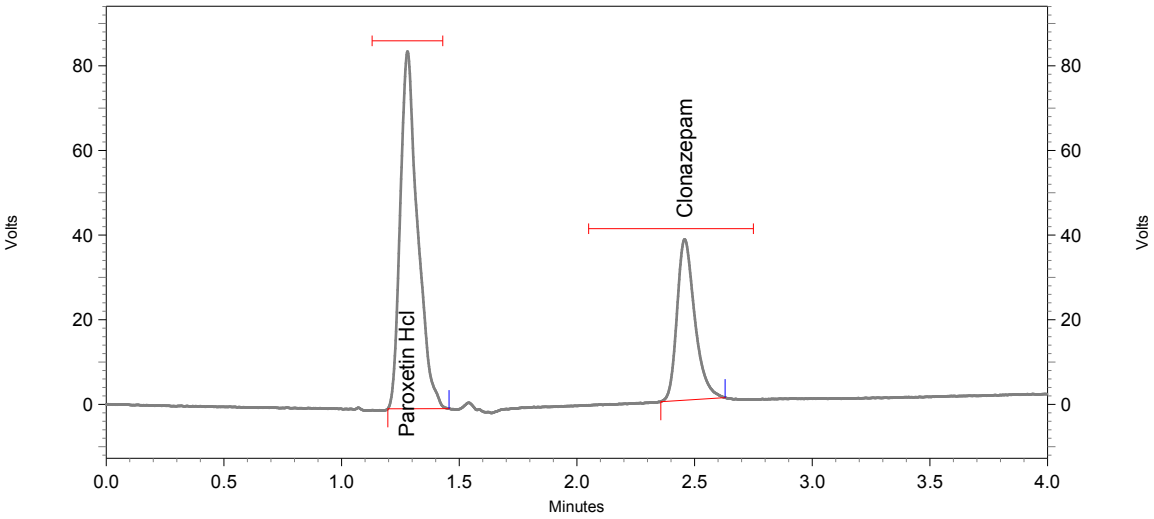
CHROMATOGRAM NO -10

System suitability 4



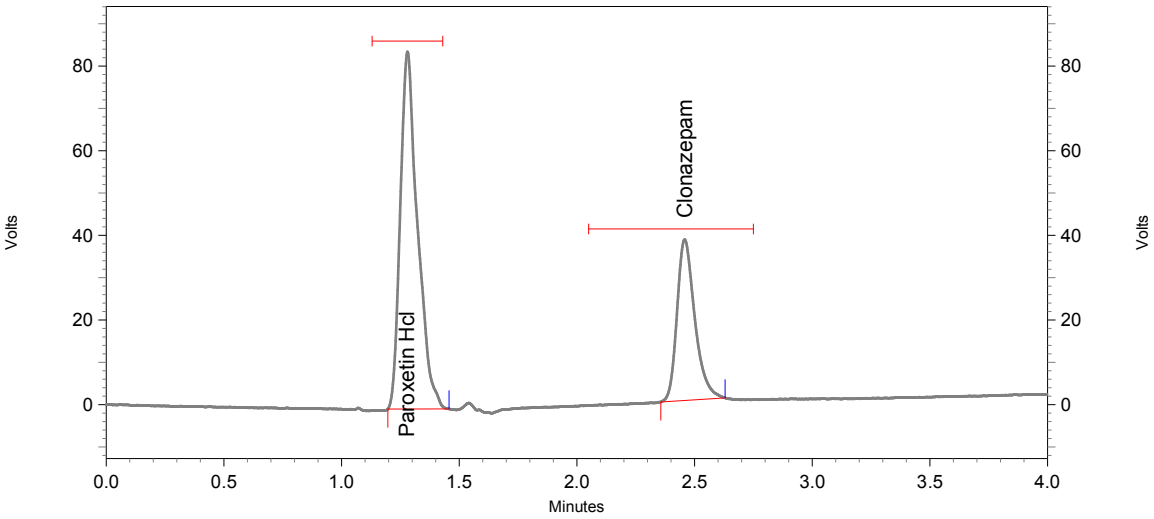
CHROMATOGRAM NO -11

System suitability 5



CHROMATOGRAM NO -12

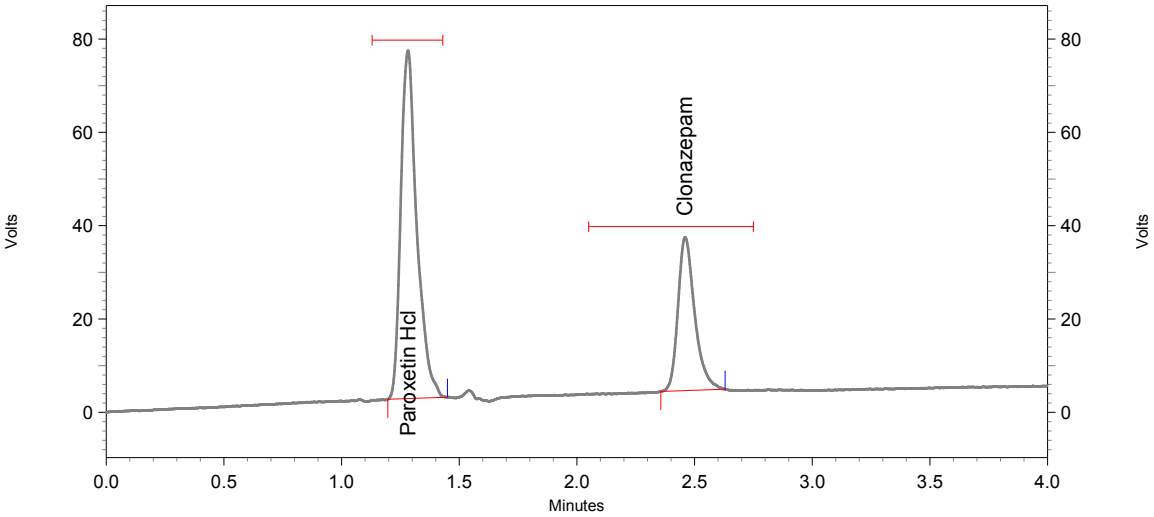
System suitability 6



LINEARITY

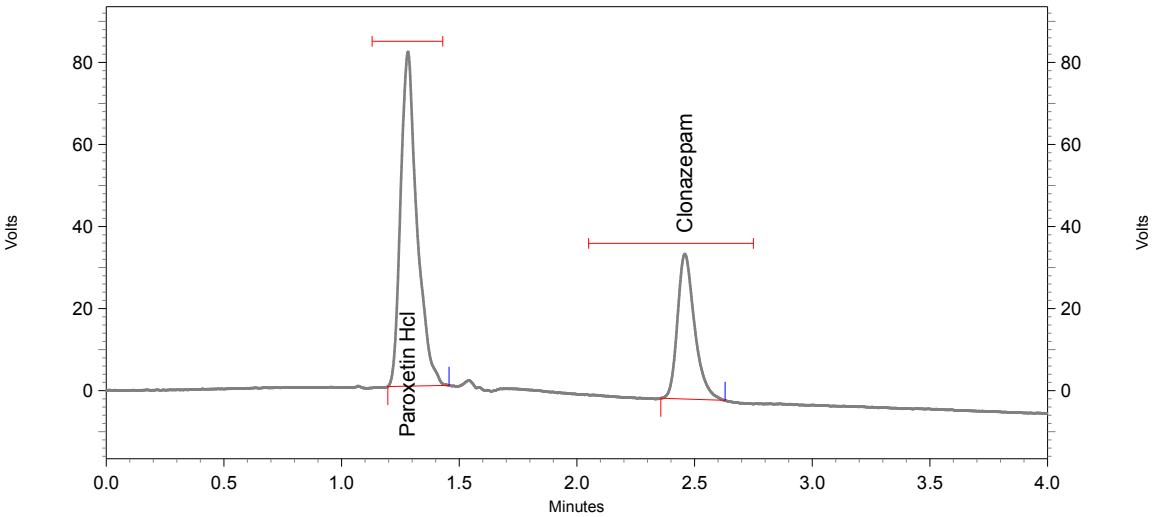
CHROMATOGRAM NO -13

Linearity 50%



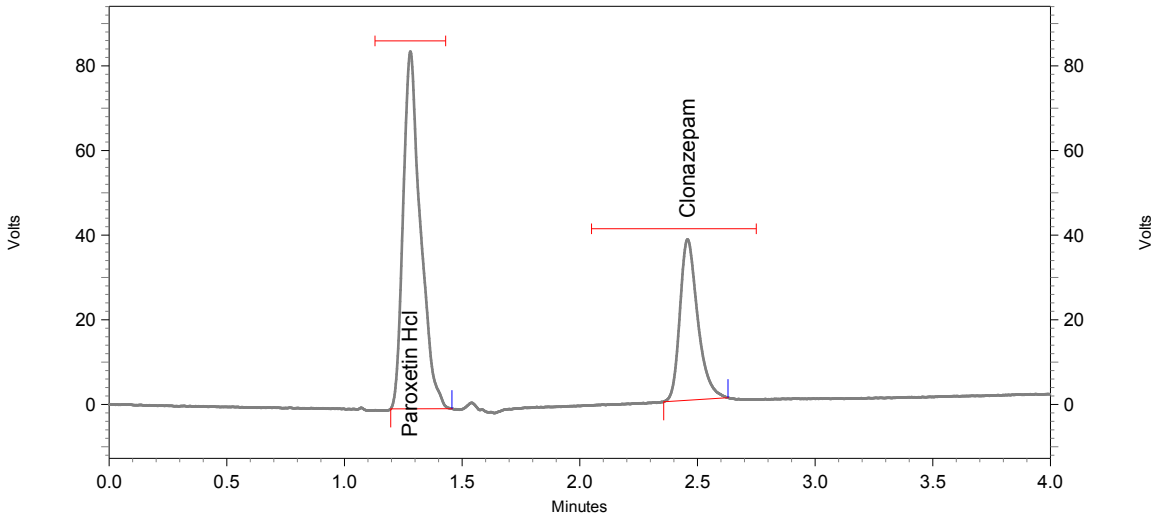
CHROMATOGRAM NO -14

Linearity 75%



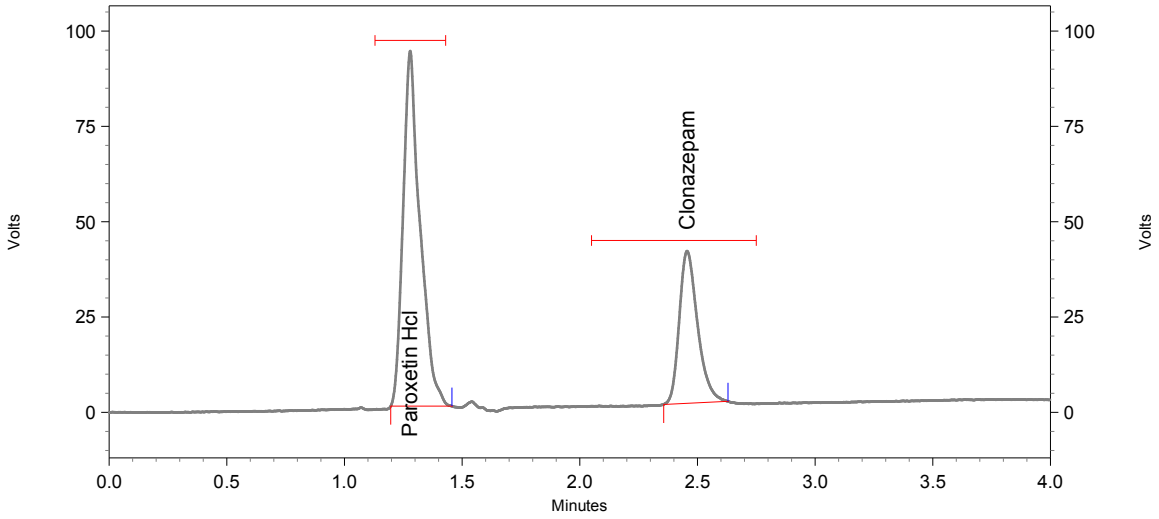
CHROMATOGRAM NO -15

Linearity 100%



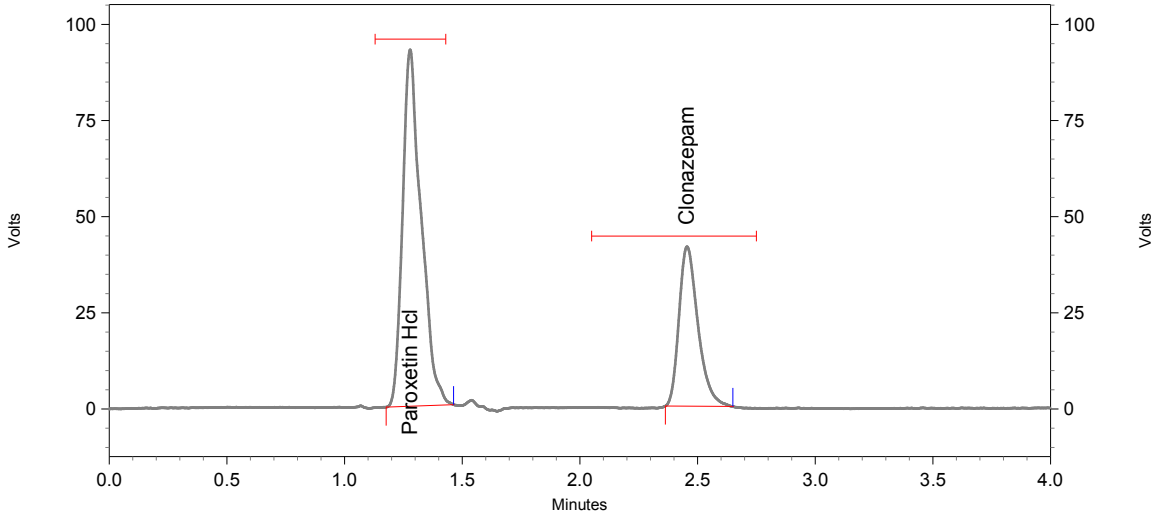
CHROMATOGRAM NO -16

Linearity 125%



CHROMATOGRAM NO -17

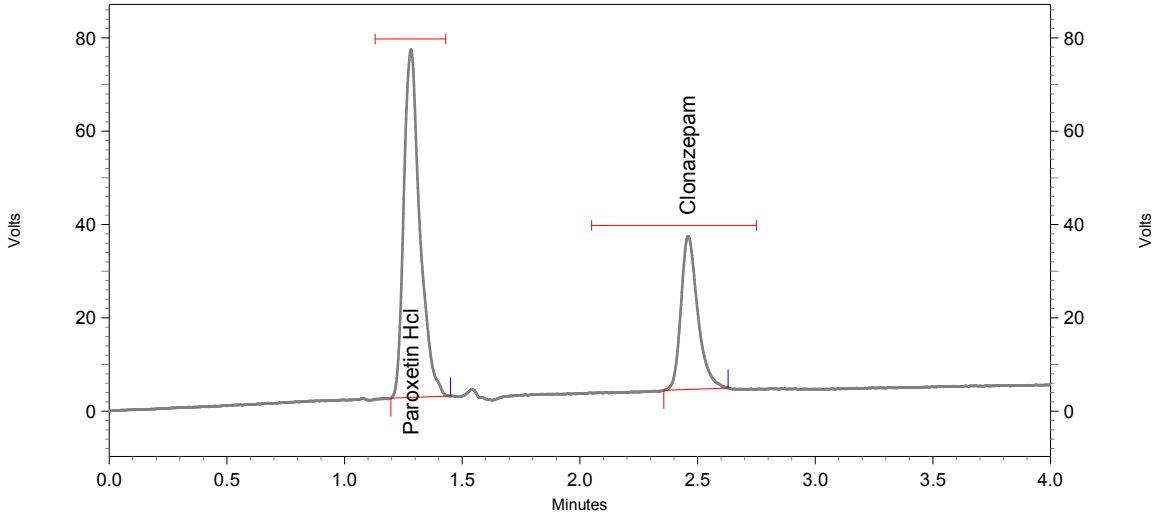
Linearity 150%



ACCURACY

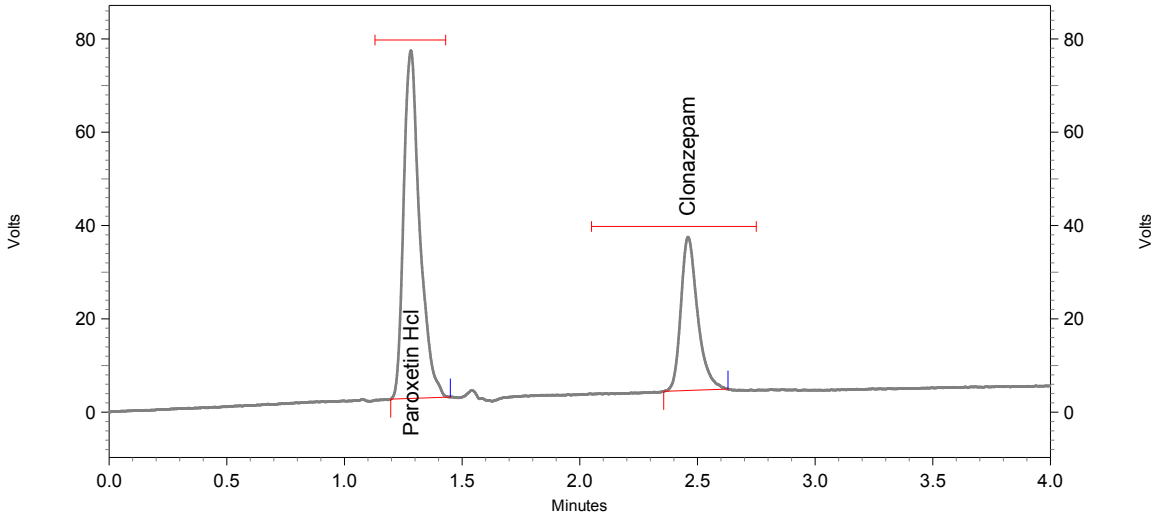
CHROMATOGRAM NO -18

Accuracy 50% [1]



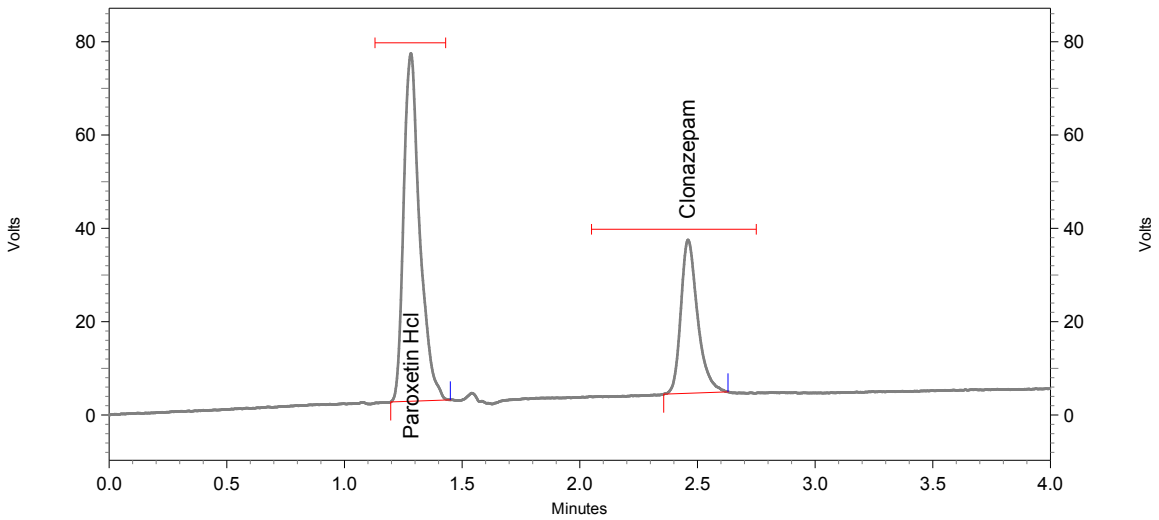
CHROMATOGRAM NO -19

Accuracy 50% [2]



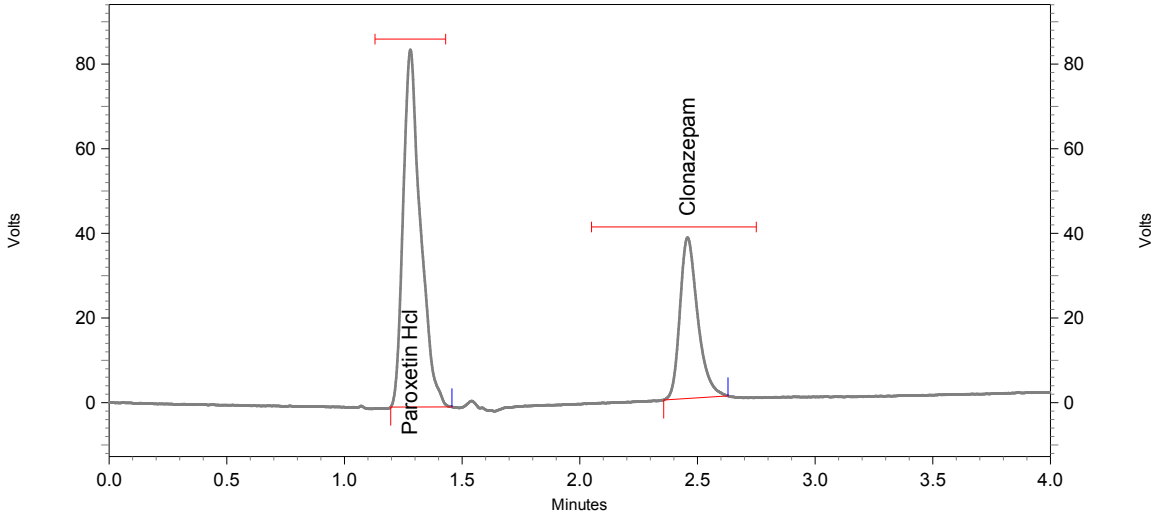
CHROMATOGRAM NO -20

Accuracy50% [3]



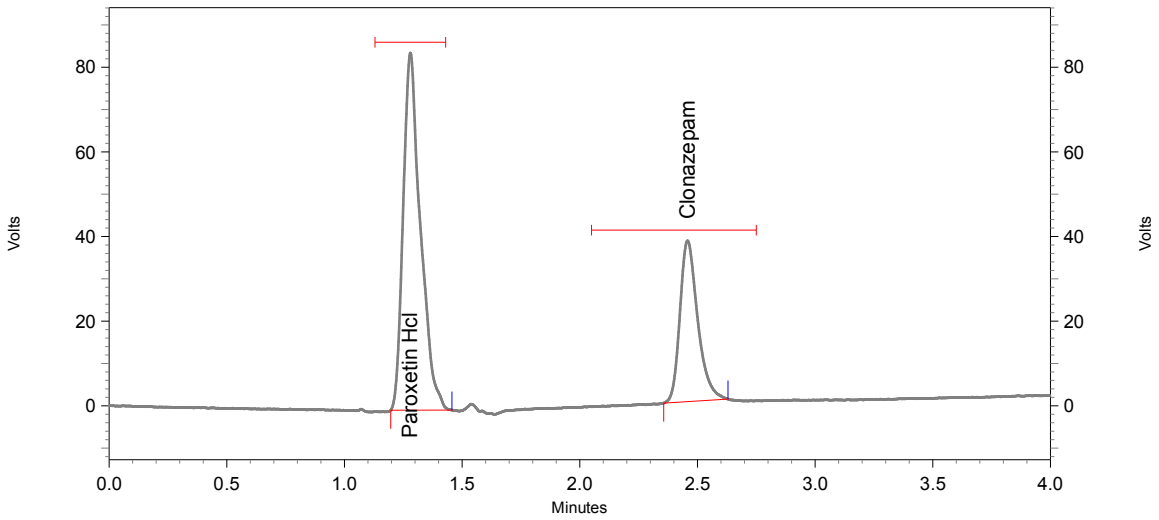
CHROMATOGRAM NO -21

Accuracy 100% [1]



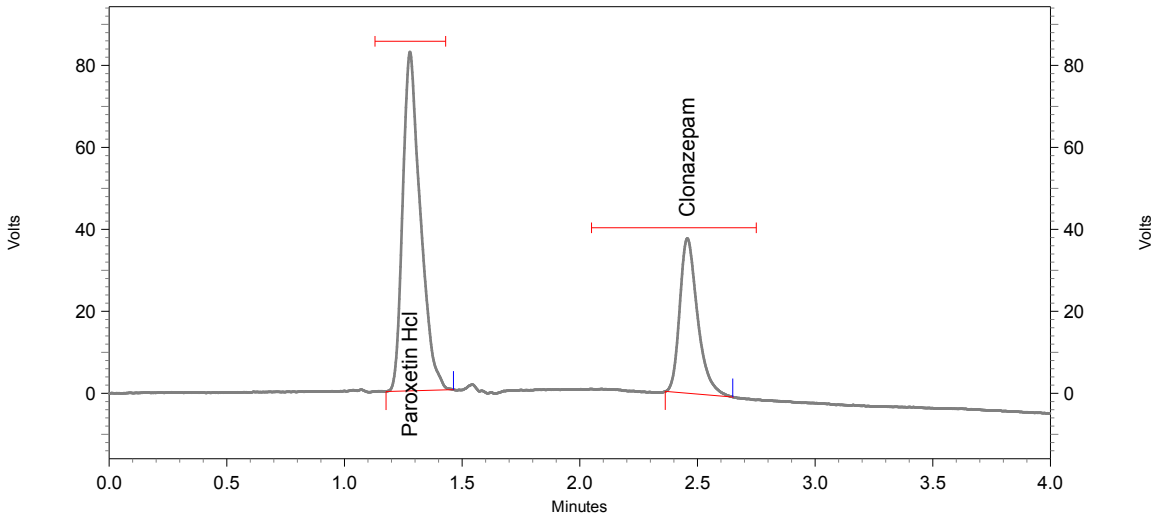
CHROMATOGRAM NO -22

Accuracy 100% [2]



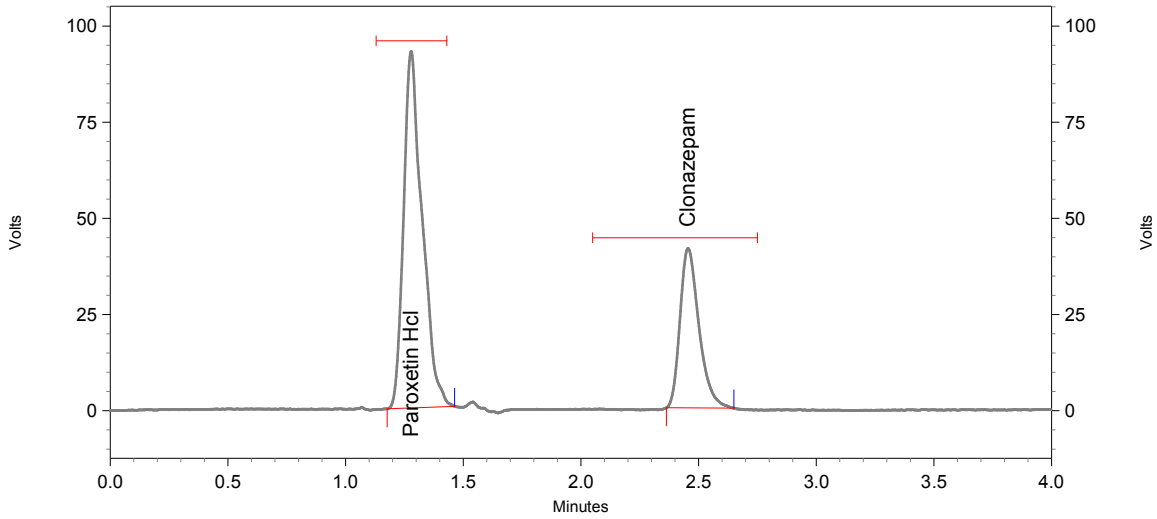
CHROMATOGRAM NO -23

Accuracy 100% [3]



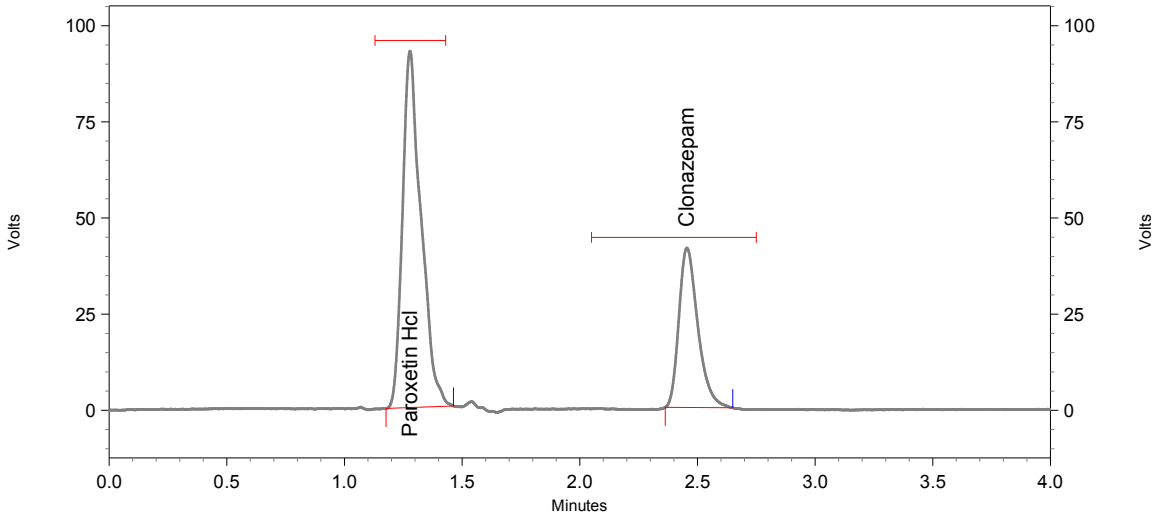
CHROMATOGRAM NO -24

Accuracy 150% [1]



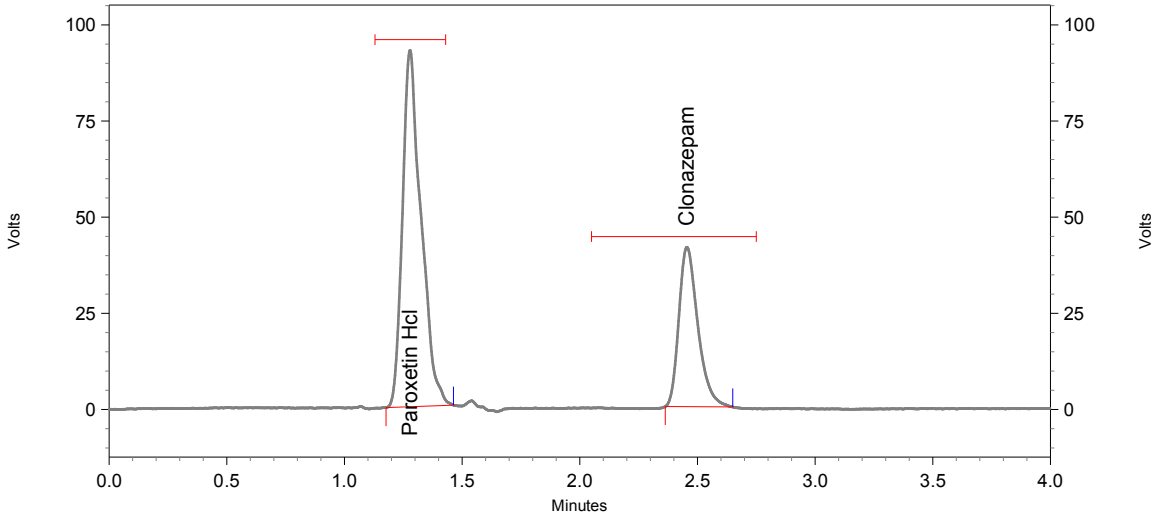
CHROMATOGRAM NO -25

Accuracy 150% [2]



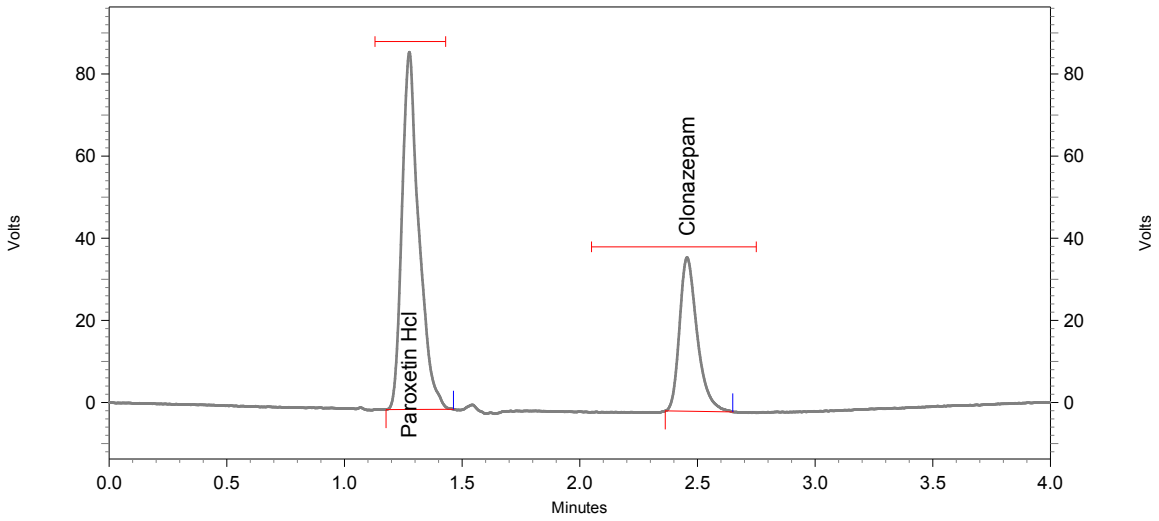
CHROMATOGRAM NO -26

Accuracy 150% [3]

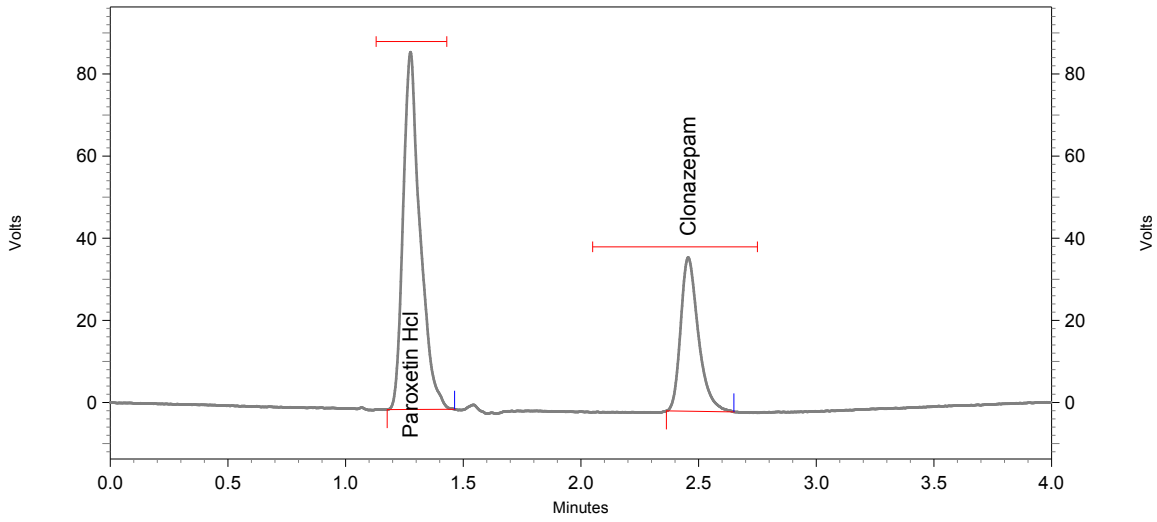


PRECISION
CHROMATOGRAM NO -27

Repeatability 1

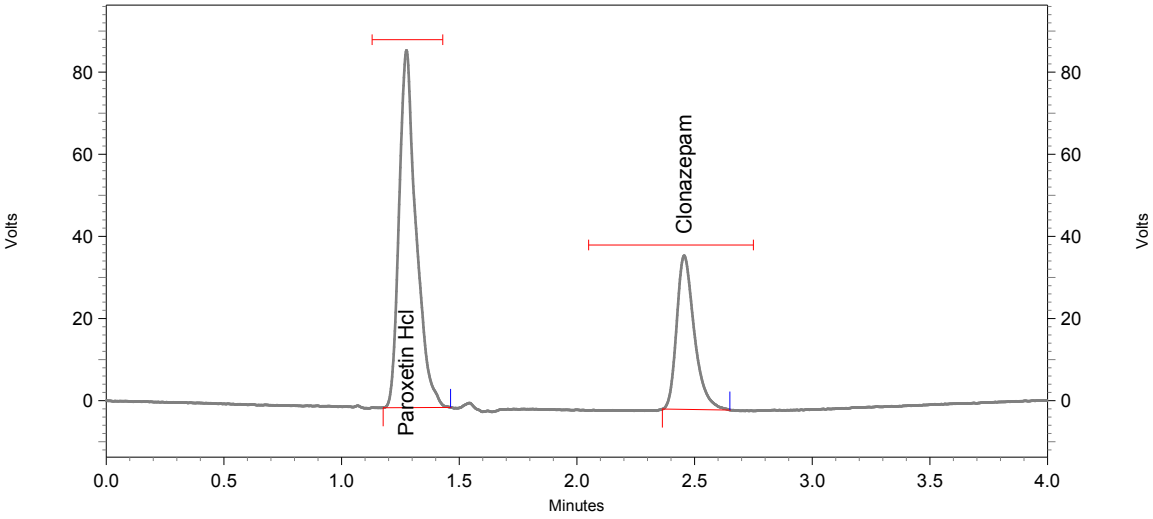


CHROMATOGRAM NO -28
Repeatability 2



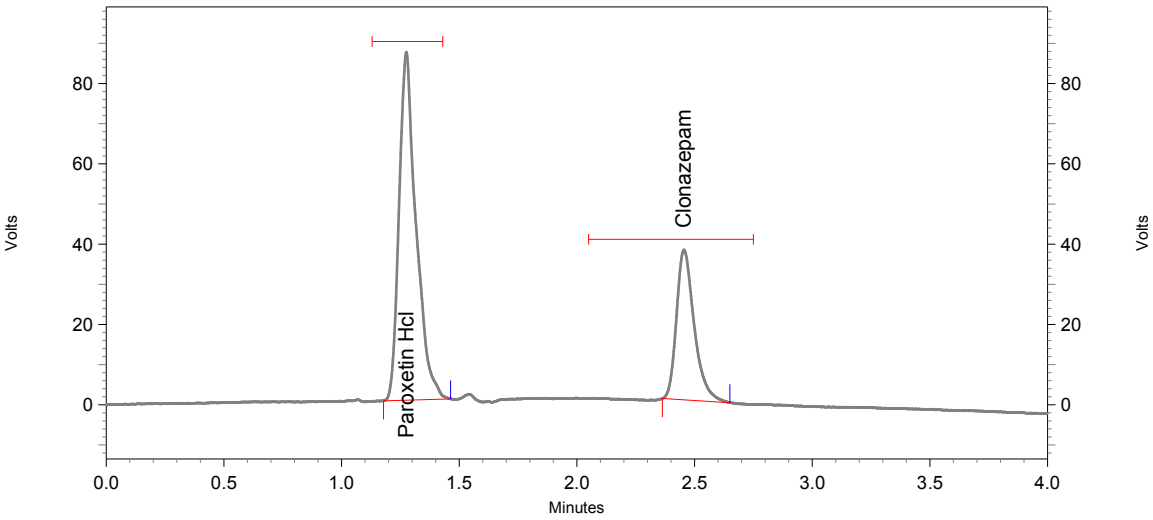
CHROMATOGRAM NO -29

Repeatability 3



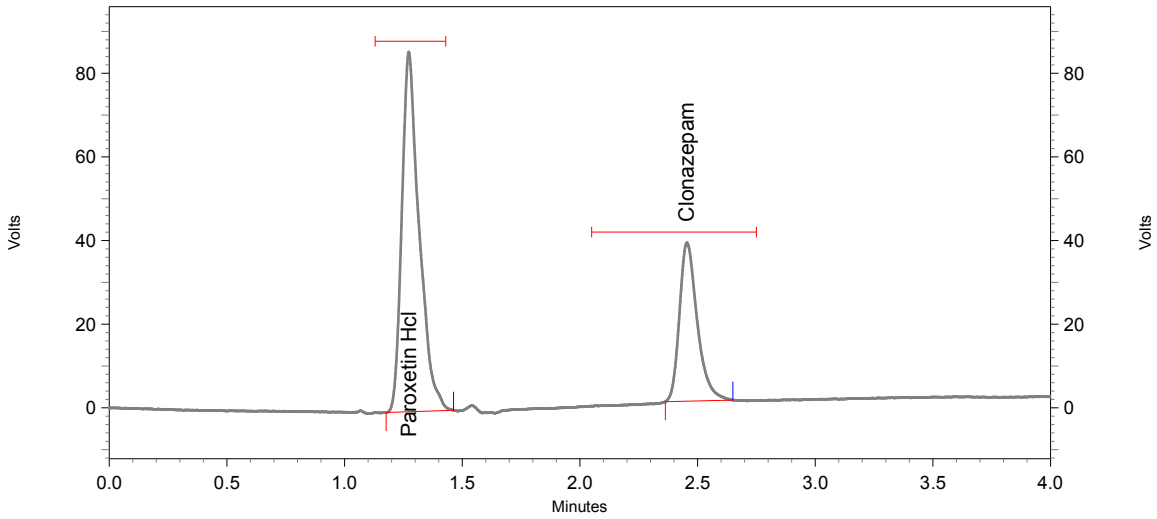
CHROMATOGRAM NO -30

Repeatability 4



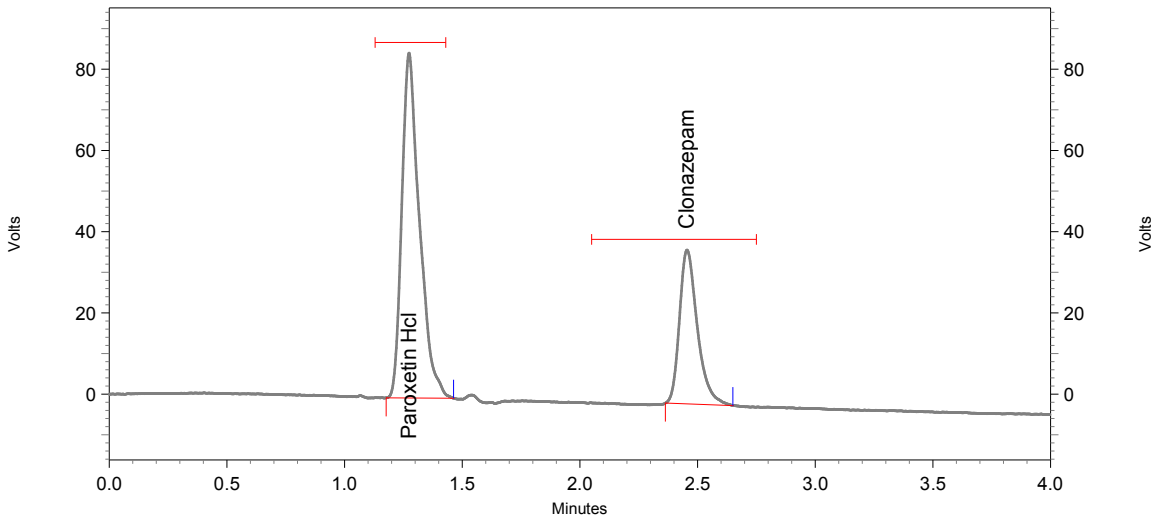
CHROMATOGRAM NO -31

Repeatability 5



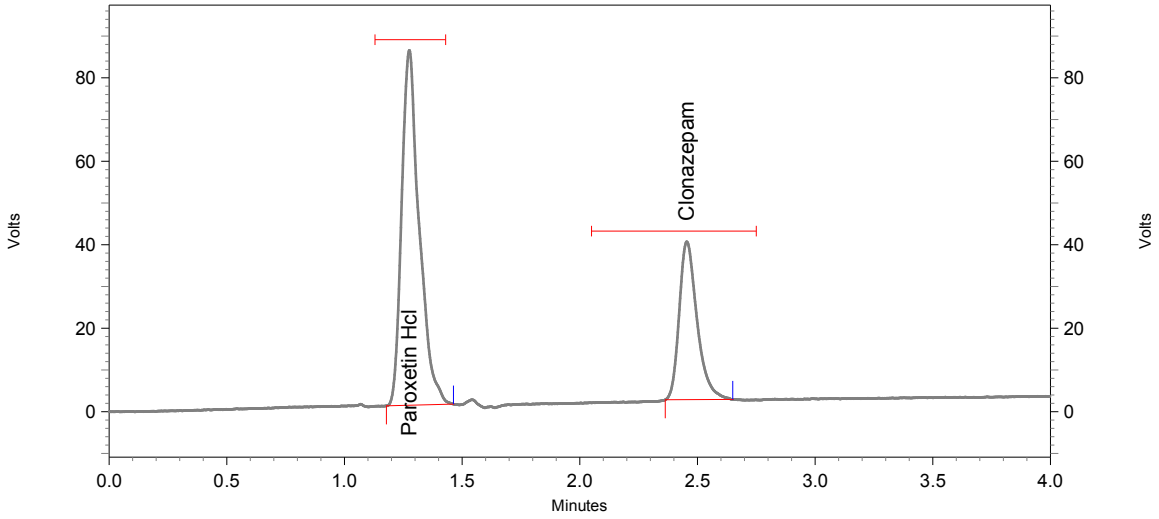
CHROMATOGRAM NO -32

Repeatability 6

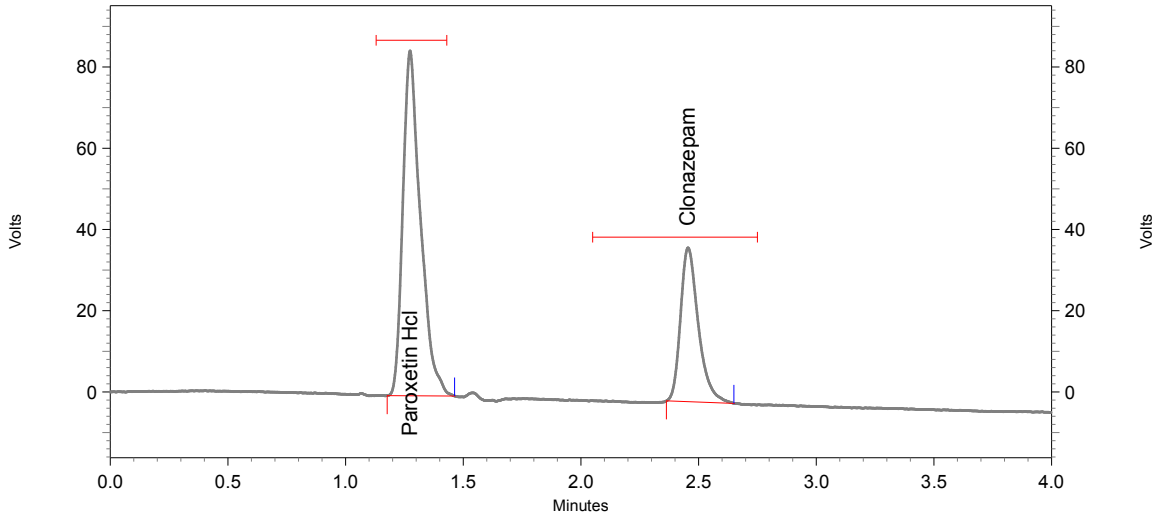


CHROMATOGRAM NO -33

Intermediate Precision[Day 1]

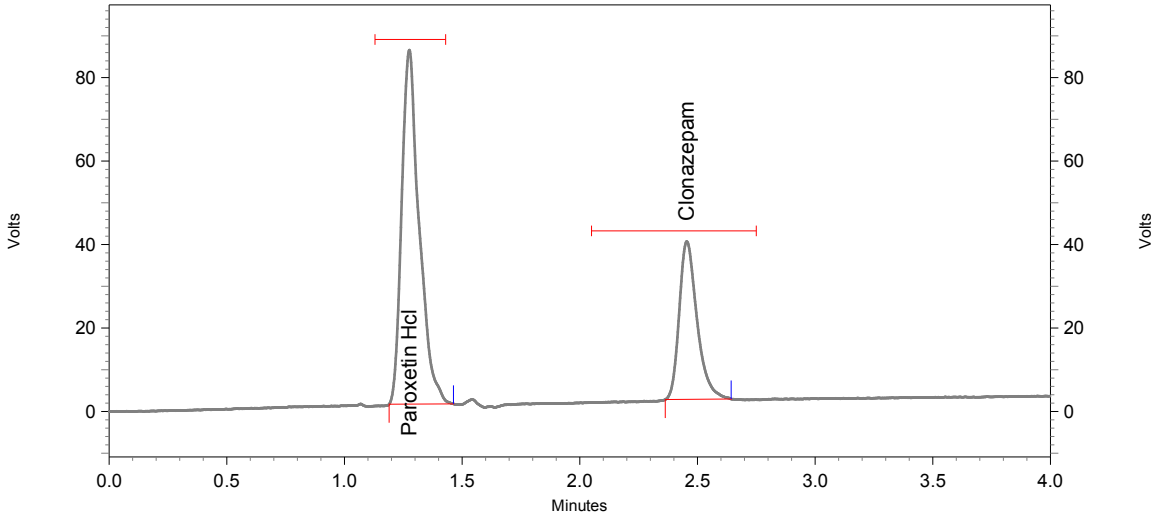


CHROMATOGRAM NO -34
Intermediate Precision [Day 2]

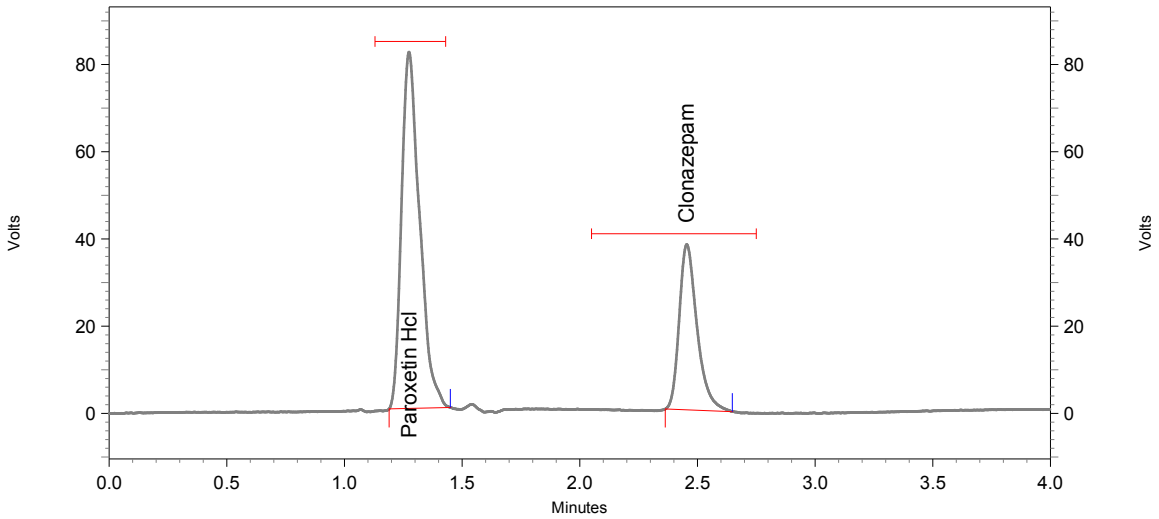


CHROMATOGRAM NO -35

Intermediate Precision [Day 3]

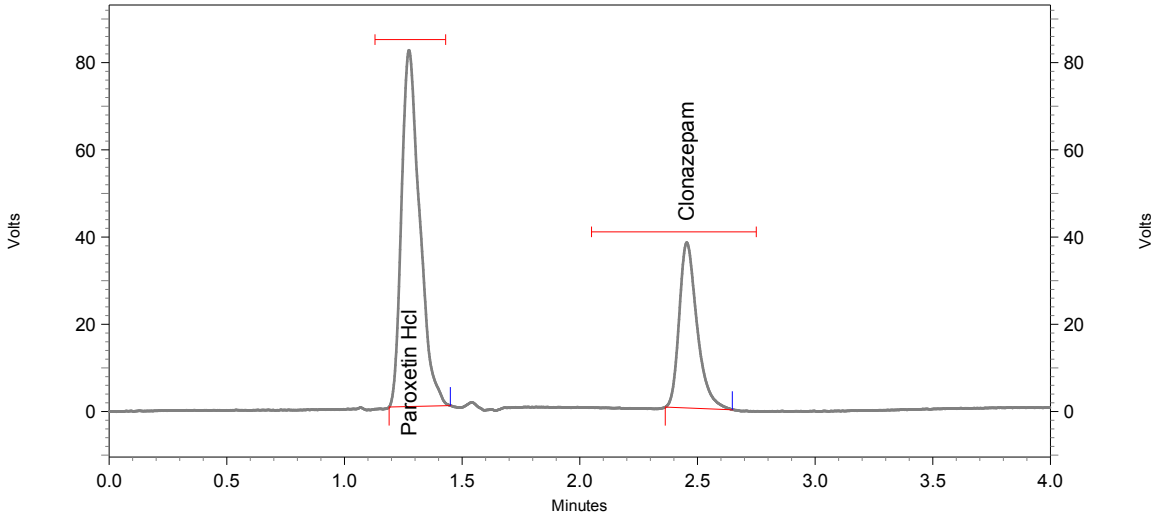


CHROMATOGRAM NO -36
Precision [Analyst 1]



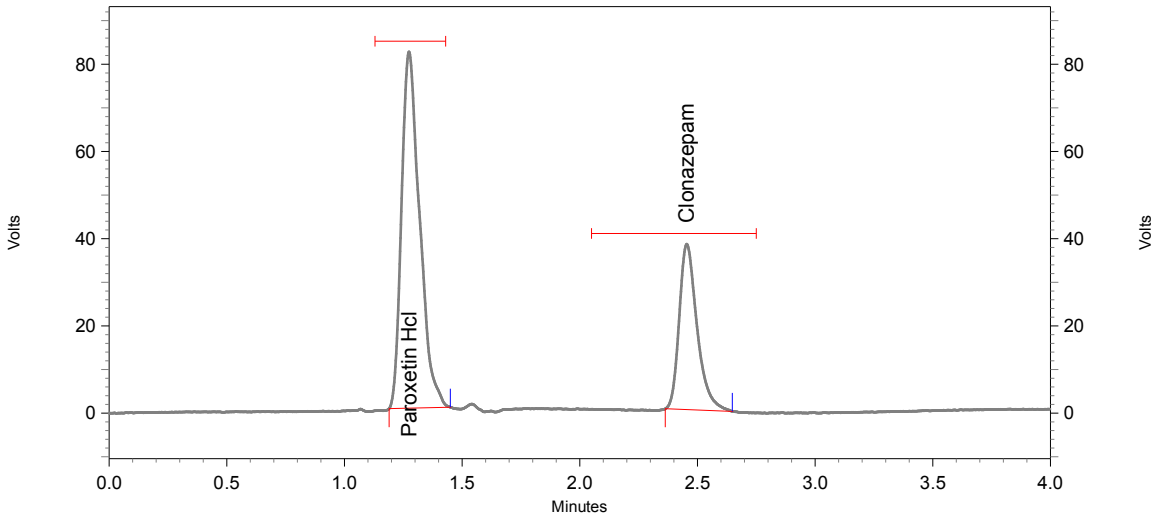
CHROMATOGRAM NO -37

Intermediate Precision [Analyst 2]



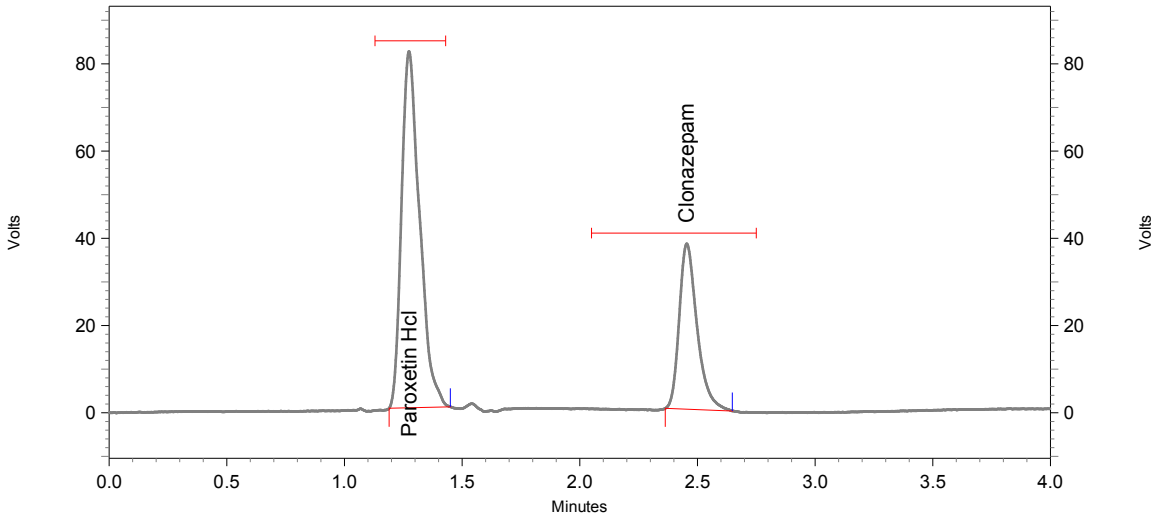
CHROMATOGRAM NO -38

Intermediate Precision [Analyst 3]



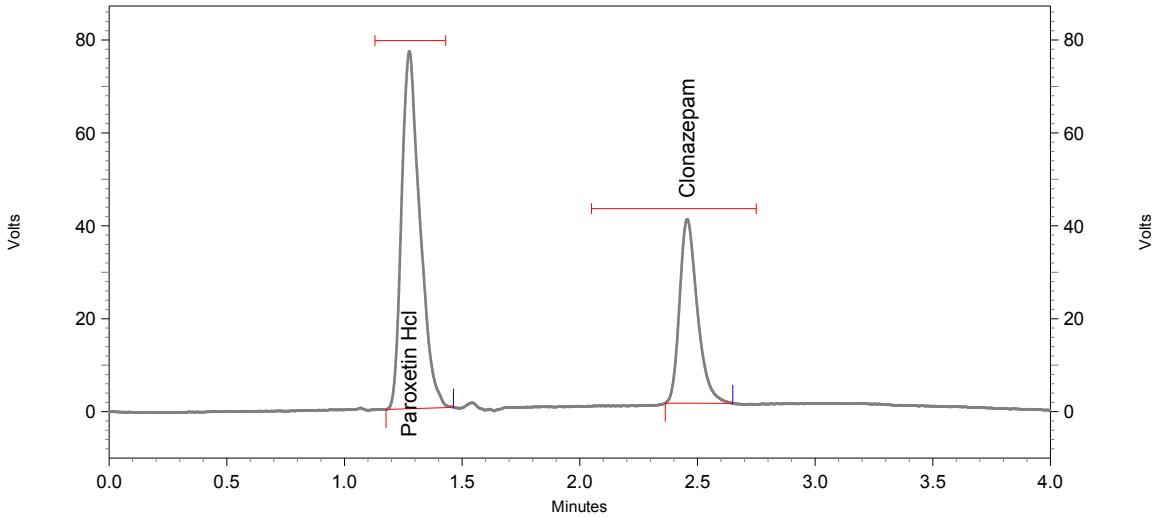
CHROMATOGRAM NO -39

Intermediate Precision [Instrument 1]



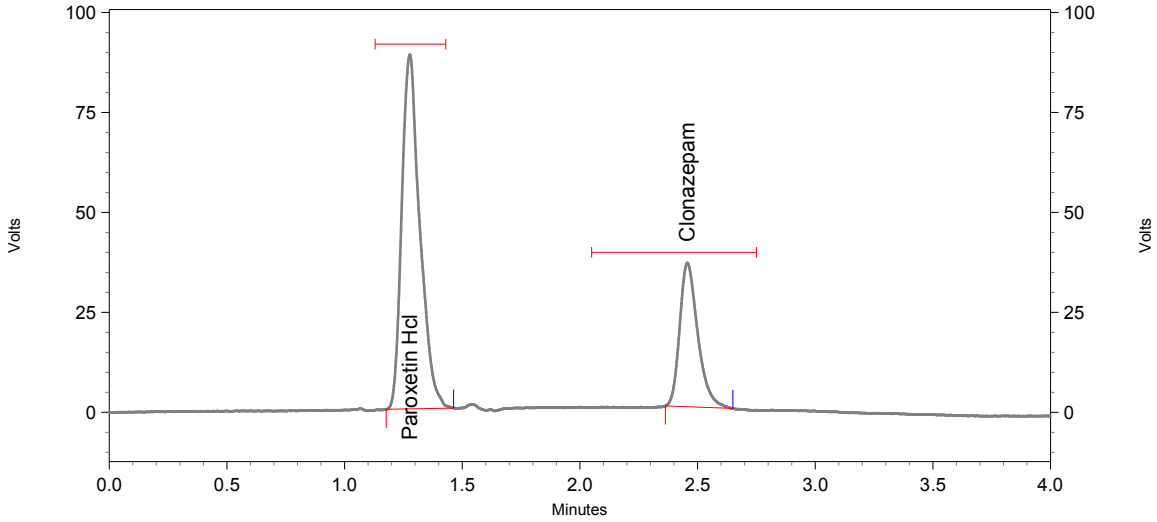
CHROMATOGRAM NO -40

Intermediate Precision [Instrument 2]



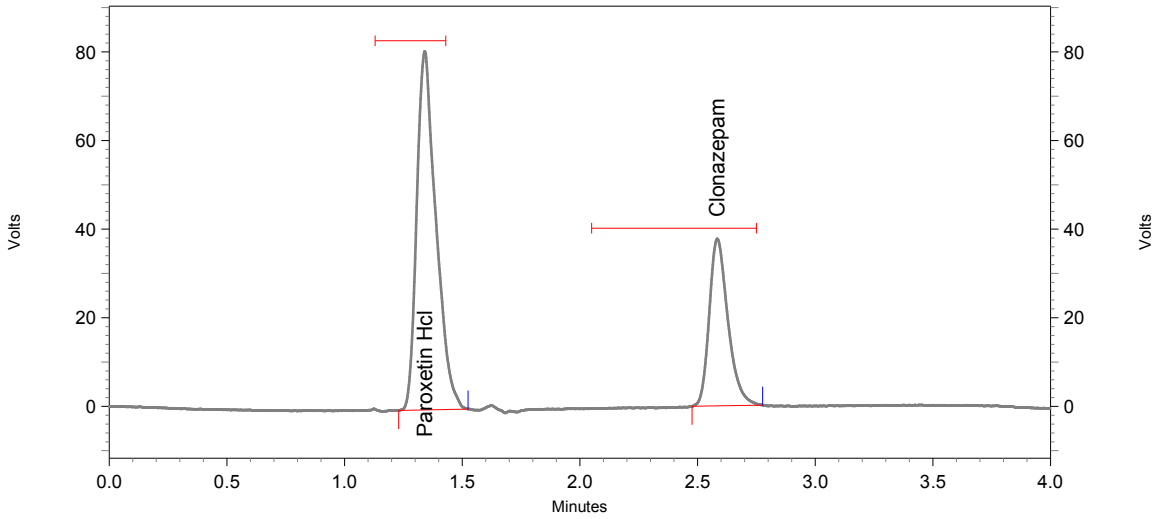
CHROMATOGRAM NO -41

Intermediate Precision [Instrument 3]



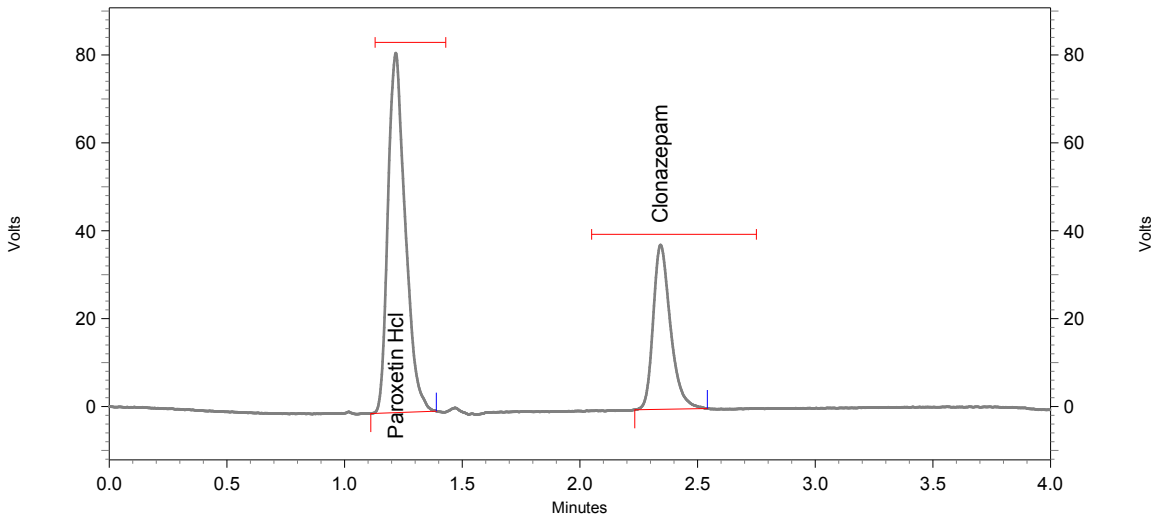
CHROMATOGRAM NO -42

Reproducibility[Lab 1]



CHROMATOGRAM NO -43

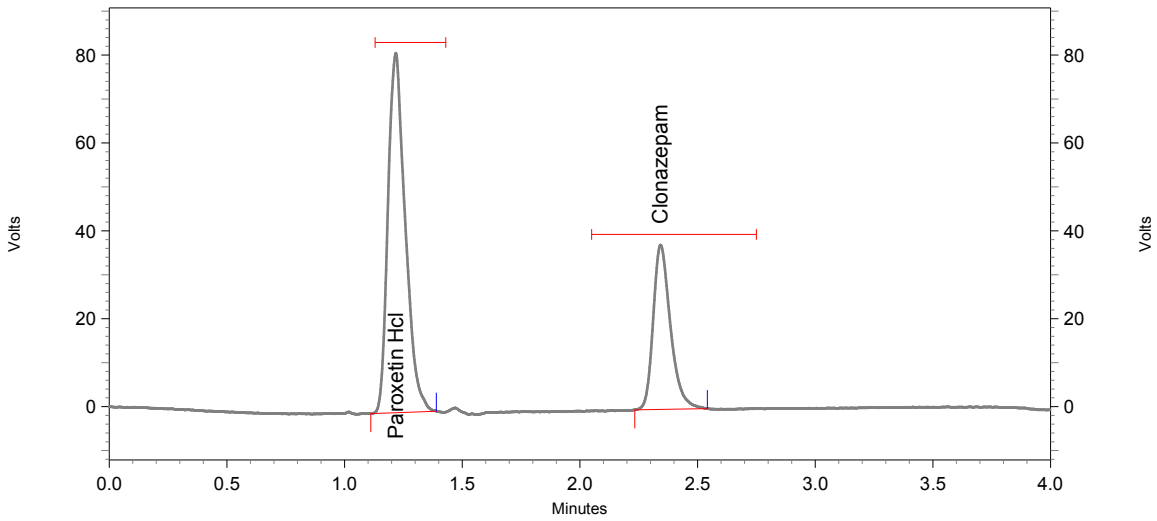
Reproducibility Precision [Lab 2]



ROBUSTNESS

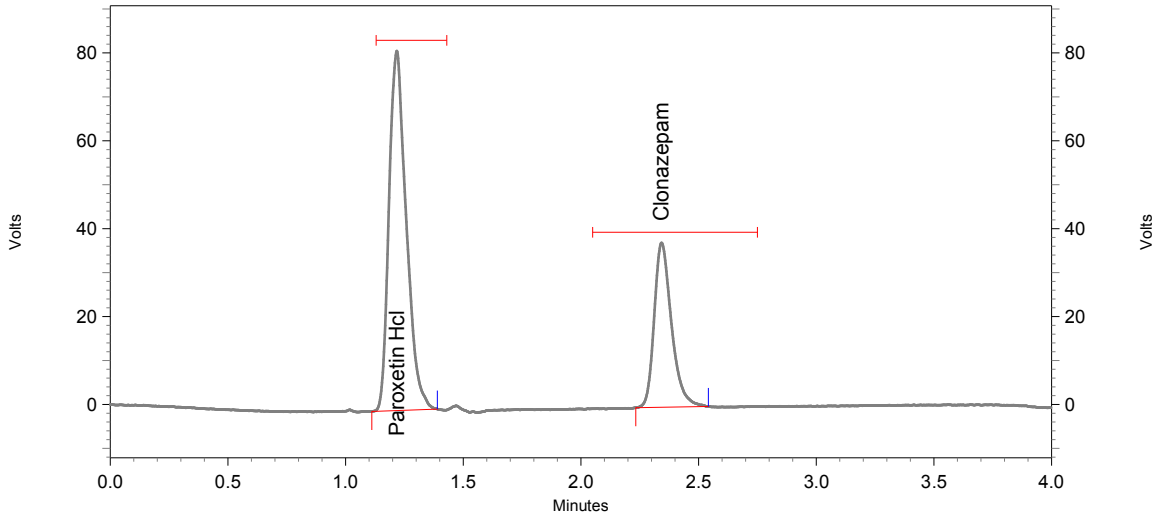
CHROMATOGRAM NO -44

Robustness (by changing wavelength +2nm)



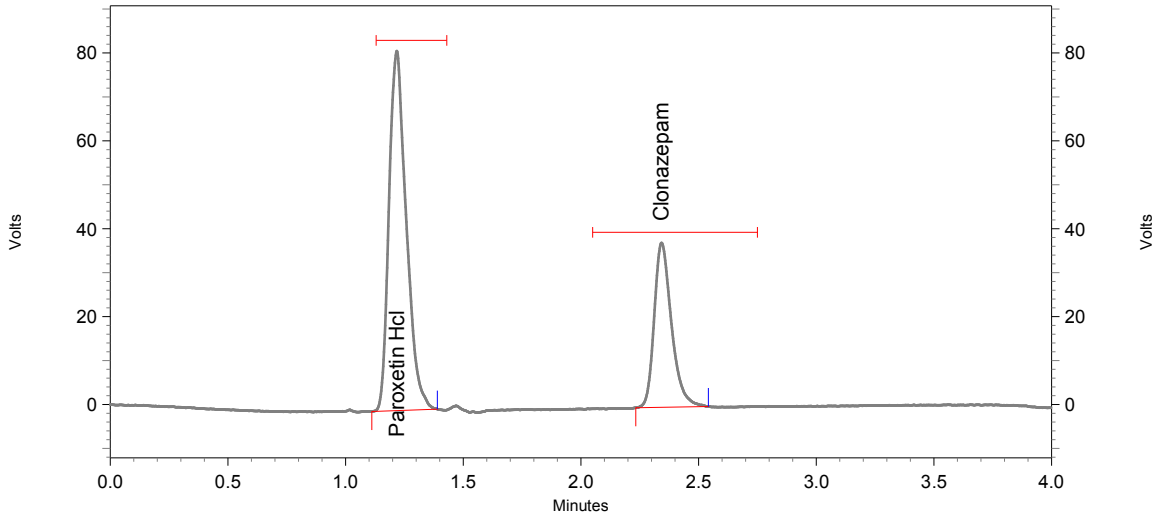
CHROMATOGRAM NO -45

Robustness (by changing wavelength - 2nm)



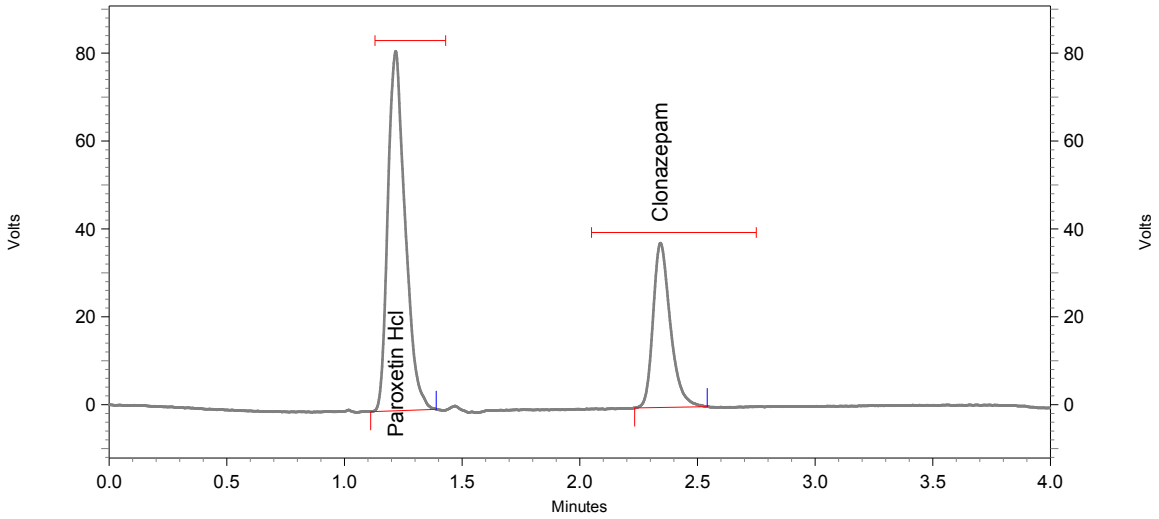
CHROMATOGRAM NO -46

Robustness (by changing flow rate + 0.2nm)



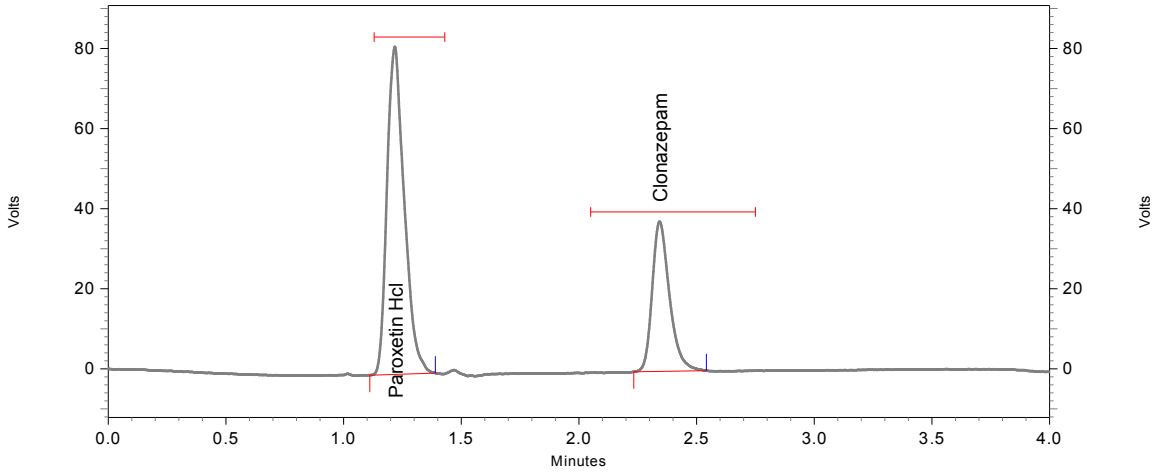
CHROMATOGRAM NO -47

Robustness (by changing flow rate - 0.2nm)



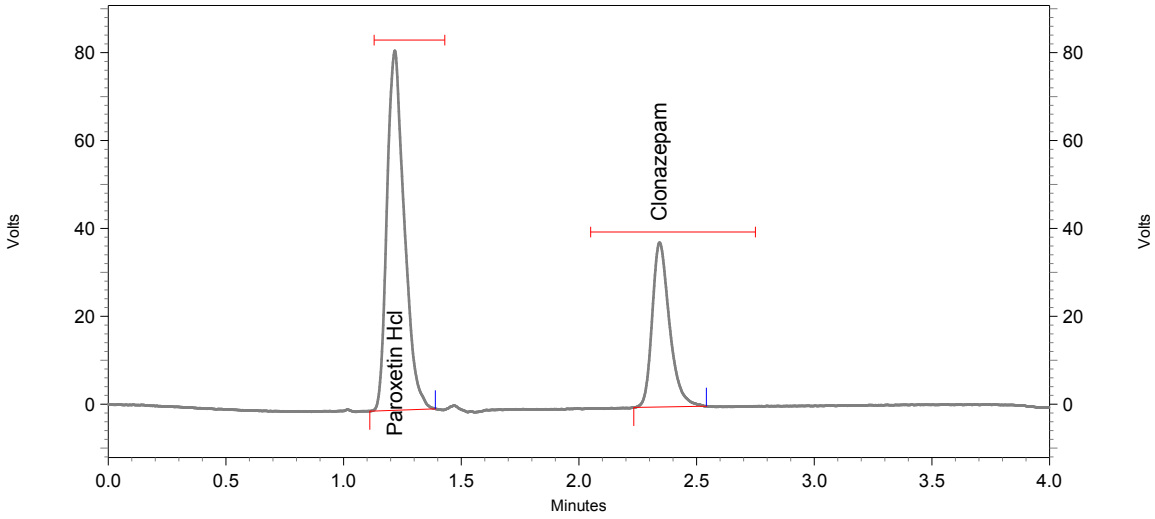
CHROMATOGRAM NO -48

Robustness (by changing mobile phase +2%)



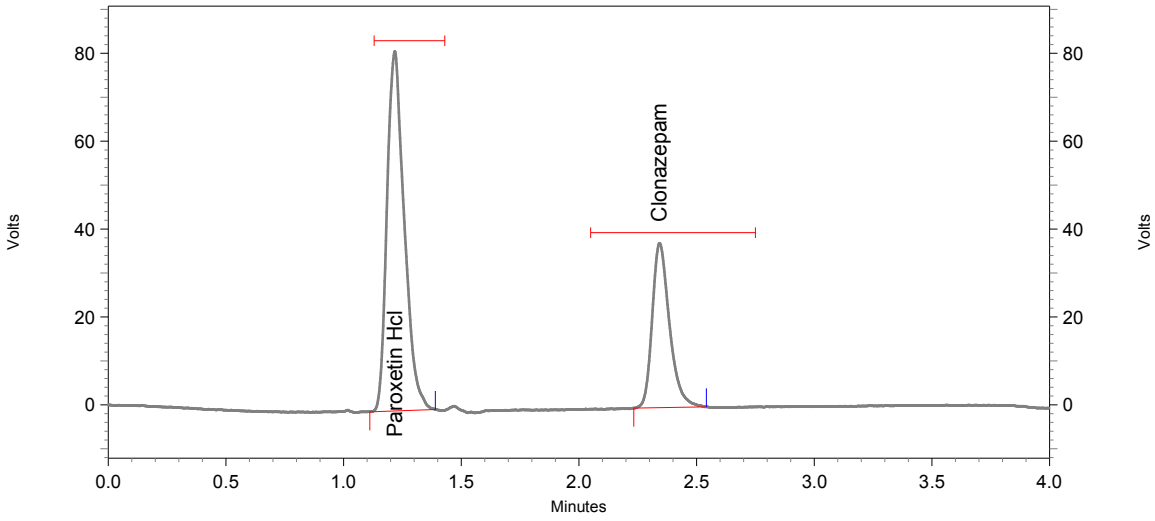
CHROMATOGRAM NO -49

Robustness (by changing mobile phase -2%)



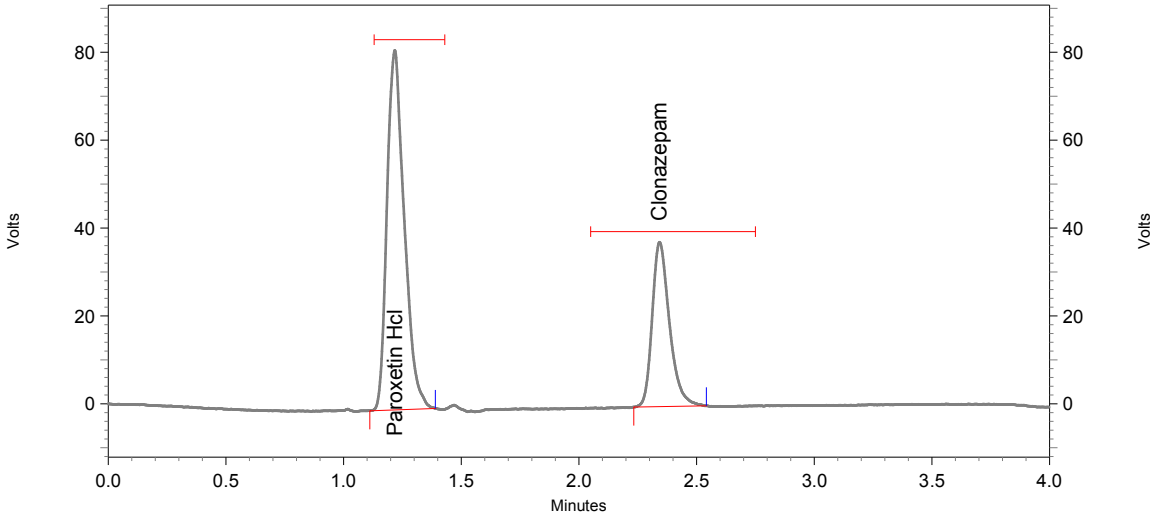
CHROMATOGRAM NO -50

Robustness (by changing pH -0.05%)



CHROMATOGRAM NO -51

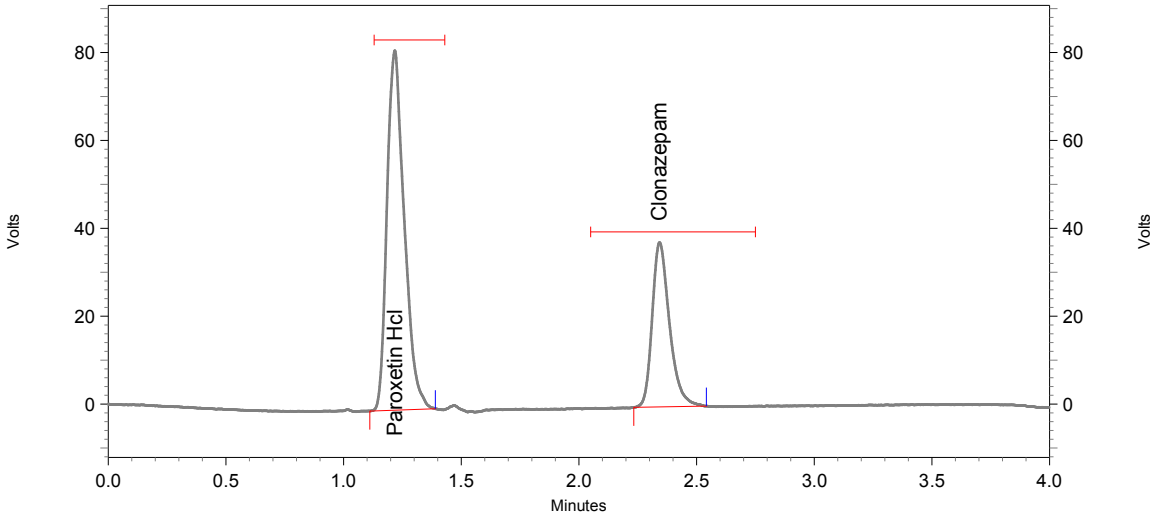
Robustness (by changing pH +0.05%)



ASSAY

CHROMATOGRAM NO -52

Assay results for commercial formulation



SUMMARY, CONCLUSION AND FUTURE SCOPE

The main objective of the present work is to develop a new simple UPLC method for simultaneous estimation of paroxetine and clonazepam combined dosage form.

Linearity of the method was established by analysis of mixed standard solution containing 250-750 µg/ml for Paroxetine and 10-30µg/ml for Clonazepam. The calibration curves drawn by plotting the response versus concentration were found to be linear and their coefficient of correlations (R^2) values are 0.9993 and 0.9997 for paroxetine and Clonazepam respectively

The good percentage recovery of the sample clearly indicates . The reproducibility and accuracy of the developed method. Similarly the RSD value for precision was found to be within acceptable limit.

Thus, to summarize, the proposed UPLC method of analysis was found to be accurate and precise, as depicted by the statistical data of analysis. The developed method is non tedious, with a very simple phase composition extremely small flow rate and relatively short run time. All these factors enable rapid quantification and simultaneous analysis of two drugs in bulk and pharmaceutical formulation without any excipient interference. It can therefore be concluded that the reported method could find practical application as an economical and rapid quality control tool for simultaneous analysis of the cited drugs from their combined dosage forms in both research and industrial quality control laboratories

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GLOSSARY

- **Degassing**: The process of removing dissolved gas from the liquid mobile phase before or during use. Degassing is done by heating by vacuum or by helium purging.

- **Drift**:The change in the baseline value over time, expressed mathematically as the slope of the least line squares line fitted to the base line in specified region chromatogram.
- **Eluent**:Mobil phase used to perform a chromatogram separation. The liquid that exist through a chromatographic column during a separation.
- **Equilibration**: The process of bringing a chromatographic solvent (solvent, column, and interactive surface) to a thermally and chemically stable state, usually indicated by drift-free base line.
- **External standard**: A separate sample that contains know quantities of the same compounds that are in unknown samples. External standards are used for quantitation by matching the detector response of a component. The calibration curve is generate from a separately processed standard (or set of standards).
- **Fronting peak**: An asymmetrically shaped chromatographic peak in which the front part of the peak (before the apex) contains more area then the rear of the peak. Teasymmetry factor for fronting peak has value less then one. The opposite of fronting peak is a tailing peak.

- **Fused peak**: Two or more no baseline-resolved peaks in a chromatogram that share the same baseline, the same baseline start and end points, and the same slope and offset.
- **Elution**: Also called as solvent programming a technique for decreasing the separation time by increasing the mobile phase strength over time during a chromatographicseparation. Gradients can be continuous or stepwise. Binary (2-solvent), ternary (3-solvent) and quaternary (4-solvent) gradients are used routinely in HPLC.
- **Integration**: The mathematical process of calculating an area such a chromatographic peak that is bounded in a part or in a whole by a curved line.
- **Isocratic**: The condition in which the solvent composition, flow rate, and the temperature are constant during a chromatographic run, the condition in which the solvent composition is constant during a chromatographic run.
- **Mobile phase**: The fluid (gas or liquid) that carries solutes through a chromatographic column. In LC, the liquid that is pumped through fluid path of the chromatographic system and into which the samples are injected.
- **Plate Count**: A measure of the observed chromatographic resolution based on its equivalency to the number of theoretical plates that would provide the same resolution.
- **Tailing Factor**: A measure of peak symmetry, where a symmetrical peak has a tailing factor of 1. As tailing increases, peak symmetry increases for system suitability, the tailing factor is the width of the peak at 5% height, divided by two times the distance from the peak maximum to the leading edge of the peak

(where the distance is measured at point 5% of point the peak height from the baseline).

- **Acceptance Criteria**: Numerical limits, ranges, or other suitable for acceptance of the results of analytical procedures.
- **Drug Product:** A finished dosage form, for example, a capsule tablet, or solution that contains drug substances, but not necessarily, in association with one or more other ingredients.
- **Drug Substance/Active Ingredient:** An active ingredient that is intended to furnish pharmacological active or other direct effect the structure are any function of the human body. The active ingredient dose not includes intermediates used in the synthesis of such ingredient. The term includes those components that may undergo chemical change in the manufacture of the drug product
- **Reagent:** For analytical procedures, any substance used in a reaction for the purpose of detecting, measuring, examining, or analysing other substances.
- **Specification:** The quality standards (i.e., tests, analytical procedures, and acceptance criteria) provided in a approved application to confirm the quality of the drug substances, drug products, intermediate, raw materials, reagents, and other components including container closure systems, and in-process materials.
- . **Spiking:** The addition of a small known amount of a known compound to a standard, sample, or placebo, typically for the purpose of confirming the performance of an analytical procedure or the calibration of an instrument.
- **Working standard:** A standard that is qualified against and used instead of the reference standard (also known as in-house or secondary standard).